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ANTHOCYANIN PRODUCTION IN DETACHED PETALS OF IMPATIENS BALSAMINA L.[1,2]

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INTRODUCTION

Anthocyanin biosynthesis in plants appears to be under manifold controls. Genetic capacity for pigment production must be complemented by a sequence of rather definite developmental events and proper environmental and nutritional factors. While biochemical pathway analysis is successfully tying flavonoid synthesis to the major processes of cellular metabolism, many key physiological control mechanisms appear obscure. With the exception of intensive investigations by the Beltsville group on the light requirement for anthocyanin synthesis, there are few satisfactory accounts in the literature of the mode of action of such external influences as nutrition, temperature, age or injury. Because anthocyanin coloration takes place only under specific circumstances it is potentially an easily recognized indicator of the metabolic state of cells producing it. Anthocyanins can assume such a role only after specific controlling reactions along their biosynthetic paths have been causally related to specific cellular events. This study defines a system in which such causal connections may be detected and provides indications of some of the controlling reactions.

Petals of *Impatiens balsamina* L. were chosen as material for these investigations. Recently Alston and Hagen (1) described the genetic control of anthocyanin production by these petals and subsequently Clevenger (4) provided similar information for the flavonols present. Petals are gaining favor as suitable organs for studying several developmental phenomena, notably protein synthesis during cell expansion (11), and metabolism of senescence (19), as well as for studying specific metabolic and biosynthetic relationships (20). The immediate aims of the present work were to isolate immature petals into a suitable culture medium, to observe the development of such petals and correlate the morphogenetic events with pigment production; and to examine the physiology of both developmental and synthetic steps by attempting to alter normal development by chemical intervention.

MATERIALS & METHODS

Plants of the garden balsam (*Impatiens balsamina* L.) were grown in the greenhouse in gravel with daily watering and weekly application of a soluble commercial fertilizer. The genotypes employed were 11hhP^{r}P^r (pink), 11HHP^{r}P^r (red), and LLhhP^{r}P^r (purple) as designated by Davis et al (5). The red and purple flowers are intensely pigmented while pink is present only as a dilute color. Buds were collected, weighed, sterilized in a 1% sodium hypochloride solution for 15 minutes, rinsed, and dissected. The four inner petals from about 20 buds were floated on sterile water. Twelve petals, picked at random, were transferred to 25 ml Erlenmeyer flasks containing 5 ml of the medium described below. This gave a petal equivalent of three flowers per flask. The culture medium was White's nutrient (22) with the modification that iron was supplied as Fe-sequestrine, and copper and cobalt were included at 0.001 and 0.01 mg/l, respectively. Unless otherwise noted the nutrient contained 20 g of sucrose per liter. Subsequent

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culture was at 22° C under continuous illumination of 4,000 lux provided by warm white fluorescent tubes supplemented with 100 W incandescent bulbs. At the conclusion of the experiments the petals were rinsed, blotted, weighed, and quickly frozen in stoppered vials. Pigments were extracted at room temperature with 1 % hydrochloric acid in 95 % ethyl alcohol (v/v) until petals were colorless. In the tables which follow, anthocyanin concentration is usually expressed as optical density (OD) of a solution containing the pigments of one flower equivalent in 10 ml of solvent. Absorption was measured in a Beckman DU or Spectronic 20 spectrophotometer in the region of maximum absorption (510–550 mμ), the specific wave length depending on the genotype. The extracts were concentrated under reduced pressure for chromatography. Two dimensional separation was accomplished with two solvents in the following order: *tert*-butanol, acetic acid, water (3:1:1), descending; 10 % acetic acid ascending. A spray composed of 2 % aluminum chloride in 95 % ethyl alcohol was used to stabilize spots. Papers were examined by fluorescent and ultra violet light in air and in ammonia fumes. For isolating and spectrophotometrically characterizing glycosides, and preparing aglycones, extracts were streaked on Whatman no. 3 paper which was developed with the *tert*-butanol solvent. The resulting bands were eluted with Harborne's elution mixture and rechromatographed (9). Hydrolysis of glycosides was accomplished by boiling in 20 % hydrochloric acid (w/v) for 3 to 4 minutes. The aglycones were then extracted with a few drops of iso-amyl alcohol and compared chromatographically with known pigments in at least two solvents: acetic acid, hydrochloric acid, water (30:3:10) and the *tert*-butanol solvent described above. Numerical data reported are based, in general, on values obtained from three flasks containing 12 petals each, or the equivalent of 9 flowers.

Results

Young buds of *I. balsamina* are well suited for organ culture. The four showy lateral petals are tightly enclosed in an envelope consisting of the single sepal and the fifth petal. This arrangement allows for easy surface sterilization prior to dissection. Excised petals remain afloat on a liquid medium and apparently resume development with a somewhat accelerated time course. Preliminary experiments showed that White's medium (22) without significant additions supports the growth of petals for as long as 40 days without transfer. This medium was used throughout the investigation. The amount of sugar in the medium had a marked effect on development and anthocyanin production (table I). Although immature petals are pale green in color, carbohydrate production by photosynthesis must fall far short of the requirements for normal development. Apparently, however, no other major organic chemical contribution by the rest of the plant is required for continued growth and pigmentation. Indole acetic acid in the range of 0.005 to 0.01 mg/l did not influence the state of petals in culture. Light was found to be essential for both expansion and pigment production. Cultures which were kept in total darkness for 22 days developed only a small band of red coloration adjacent to the cut basal end. Such petals also showed arrested development but were capable of resuming growth when exposed to light.

The only reliable criterion for judging the physiological age of petals is the fresh weight of buds from which they are excised. Cultures were started as a rule with petals from buds weighing 30 to 50 mg. The fresh weight of the four petals at this stage was about ten mg. Petals of a bud on intact plants start anthocyanin production when their combined weight reaches about 40 mg, at which time total bud weight is 100 mg. When petals are fully mature they weigh ca. 400 mg per flower if left on the plant and about 150 to 300 mg if cultured. The increase in fresh weight probably represents expansion only because even the youngest petals cultured had already assumed their final shape. That weight may be directly correlated with area was demonstrated by the constancy of the ratio fresh weight/surface measured for three flasks, a total of 36 petals. The average value was 0.21 mg/mm² with a narrow range of variation.

Time Course in Culture. The time course of expansion and anthocyanin synthesis is summarized in figure 1. These curves show that expansion commences immediately and continues until about the 13th day when a sharp increase in the rate sets in. In general, anthocyanin synthesis follows the same pattern as expansion with both activities beginning to level off about 20 days after culturing.

Modification of Pigment Pattern by Culturing — Quantitative Aspect. Quantitative comparison of anthocyanins produced by detached and undisturbed petals is presented in table II. In the intensely colored flowers of 11HHP^{r}P^r and LLhhP^{r}P^r anthocyanin content of cultured petals approximates the values obtained from intact flowers. In the dilute pink line of genotype 11hhP^{r}P^r, however, cultured

Table I

Effect of Sucrose Concentration on Fresh Weight & Anthocyanin Content of Detached Petals*

Sucrose conc % (w/v)	Fresh wt/ flower mg	Anthocyanin content	
		OD/ Flower**	OD/ g Tissue***
0.00	35	0.016	0.046
0.50	115	0.235	0.204
1.00	135	0.411	0.305
2.00	174	0.575	0.330
2.50	177	0.612	0.346
3.00	182	0.575	0.317

* Genotype 11hhP^{r}P^r
** In 10 ml
*** In 100 ml

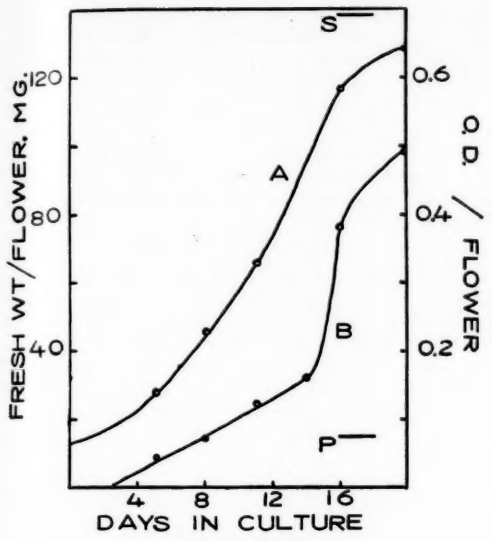

FIGURE 1.

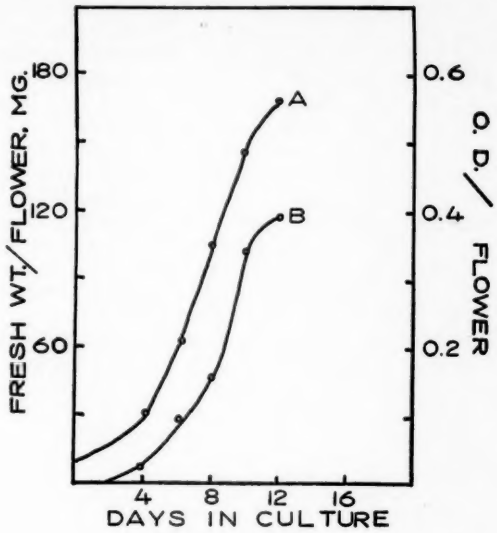

FIGURE 2.

FIG. 1. Time course of anthocyanin production and expansion of petals in culture. Expansion measured as fresh weight of four petals (curve A). Anthocyanin content expressed as OD (at λ max) of an extract containing the pigments of four petals in 10 ml of 95 % ethanol containing 1 % HCl (curve B). Pigment content of intact petals (P) and sepals (S). Genotype llhhP^{r}P^r.

FIG. 2. Time course of anthocyanin production and expansion of injured petals in culture. For description of intentional injury see text. Genotype llhhP^{r}P^r.

petals approximate intact sepals in their anthocyanin content. It thus appears that in this genotype culturing allows the production of larger quantities of pigments than are produced in situ.

MODIFICATION OF PIGMENT PATTERN BY CULTURING — AGLYCONES & GLYCOSIDES. The flavonoid pigments produced by detached petals are compared with those produced by intact flowers in table III. Undisturbed petals contain no anthocyanin or flavonol with a 3′4′-dihydroxylated B ring, whereas in culture all three genotypes produced cyanidin, two produced quercetin and one produced peonidin, all of these compounds having a basic 3′,4′-dihydroxy pattern on the B ring of the flavonoid nucleus. In this respect cultured petals took on some of the characteristics of sepals in the pink and red flowers. Also sepaloid in nature was the distribution of pigment in cultured petals, for veins became strongly delineated while in undisturbed petals the coloration is more evenly distributed.

To test the possibility that these departures from normal quantitative and qualitative patterns of pigment production resulted from mechanical injury or

TABLE II

COMPARISON OF ANTHOCYANIN CONTENT OF INTACT FLOWER PARTS & CULTURED PETALS

GENOTYPE	COLOR	FLOWER PART	ANTHOCYANIN CONTENT			
			INTACT FLOWERS		CULTURED PETALS	
			OD/FLOWER	OD/g TISSUE	OD/FLOWER	OD/g TISSUE
llhhP^{r}P^r	Pink	Petal	0.075	0.015	0.633	0.345
		Sepal	0.690*	0.262	...	...
llHHP^{r}P^r	Red	Petal	1.420	0.230	1.018	0.750
		Sepal	0.490*	0.130	...	...
LLhhP^{r}P^r	Purple	Petal	0.470	0.090	0.425	0.278
		Sepal	0.440*	0.139	...	...

* Values given are for four sepals.

premature exposure to white light which cultured petals received, the following simple experiments were performed. A large number of 11hhP^{r}P^r buds were pierced with a needle at the stage usually collected for culturing. The injured buds were allowed to develop in situ and the petals were examined at anthesis. In another experiment approximately 20 buds were divested of their outer envelope formed by the sepal and fifth petal and were then enclosed in a transparent film of "Saran Wrap" to reduce water loss. In this way white light was admitted to immature petals without detaching them. At the conclusion of these experiments the treated petals showed no departure from the usual, natural patterns in either the quantity, distribution, or quality of coloration. The dilute pink color of the 11hhP^{r}P^r genotype would have allowed easy visual detection of any alteration usually associated with cultured petals.

The glycosidic pattern of intact and cultured petals was found to be quite similar upon chromatographic comparison. It was of interest to observe in several developmental stages whether or not the various pigments appear in a specific order. As will be shown below, injury has the effect of accelerating development. The anthocyanin patterns of two groups of 11HHP^{r}P^r petal cultures were therefore compared. The first group of petals was allowed to develop in culture without any additional treatment while the second group received mechanical injury in the form of two needle tears per petal to increase pigmentation. When harvested after 14 days of incubation the injured petals showed a much higher pigment content than the control. In the control petals one of the two major pelargonidin glycosides (designated as Spot no. 3) was far more intense on the two dimensional chromatogram than the other (designated as Spot no. 4). In the more intensely colored, injured petals, Spot no. 4 seemed to predominate slightly over Spot no. 3. Densitometric measurements of chromatogram strips confirmed this observation. A comparison of the absorption spectra of the two eluted pigments showed that the pigment of Spot no. 3 possessed a shoulder at 440 mμ in addition to the major pelargonidin peak, while the glycoside of Spot no. 4 had no such shoulder. According to results published by Harborne (9) an E_{440}/E_{max} ratio of 0.39 is indicative of a free hydroxyl group in position 5 of the pelargonidin molecule, while a ratio of around 0.20 characterizes glycosides with a sugar occupying position 5. The E_{440}/E_{max} ratios of Spots no. 3 and no. 4 were 0.39 and 0.22, respectively. Full chemical and chromatographic identification of these spots as a pelargonidin-3-monoglucoside and a pelargonidin-3,5-diglucoside will be published elsewhere. Thus, it appears that, as has been suggested by Harborne and Sherratt (8), glycosidation proceeds in a stepwise fashion, in this case first the 3 and then the 5 hydroxyl groups reacting. Judging by the intensity and area of spots representing the pelargonidin glycosides of llhhP^{r}P^r petals, the sequence of events in that genotype is similar. Similar observations have been made in developmental studies on intact plants by Hagen (unpubl).

Experimental Control of Development & Pigmentation — Natural Sequence of Events. Further work with this petal system was designed to shed light on the relation between developmental events and synthetic processes leading to pigmentation. We shall first describe the course of pigment development in culture and then present the results of attempts to interfere with the observed pattern. The excised petals are initially pale green, tightly furled organs. Within 2 days after culture they flatten out and the portion of the claw adjacent to the cut end begins the production of anthocyanin. The red area slowly enlarges as its boundary moves toward the blade. At this time the cells in the blade adjacent to the claw begin to lose their green appearance and simultaneously start to expand. Expansion causes a slight hump to form at the border between the still-green and the white portions of the blade. The hump slowly travels across the surface acropetally until the entire petal has lost its chlorophyll and reached

Table III

Anthocyanidins & Flavonols Found in Cultured Petals & Intact Flower Parts

Genotype	Color	Part	Anthocyanidins				Flavonols		
			Pelargonidin	Cyanidin	Peonidin	Malvidin	Kaempferol	Quercetin	Myricetin
llhhP^{r}P^r	Pink	Intact Petal	+	...	...	...	+	...	...
		Cultured Petal	+	+	+	...	+	+	...
		Sepal	+	+	+	...	+	+	...
llHHP^{r}P^r	Red	Intact Petal	+	...	...	...	+	...	...
		Cultured Petal	+	+	...	...	+	+	...
		Sepal	+	+	...	...	+	+	...
LLhhP^{r}P^r	Purple	Intact Petal	...	...	...	+	+	...	+ (?)
		Cultured Petal	...	+	...	+	+	...	+ (?)
		Sepal	...	...	...	+	+	+	+ (?)

TABLE IV

EFFECT OF INJURY ON FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS

EXP.	GENOTYPE	DAYS IN CULTURE	FRESH WT/FLOWER mg			ANTHOCYANIN CONTENT OD/FLOWER		
			CONTROL	INJURED	% INCREASE	CONTROL	INJURED	% INCREASE
I–80	LLhhP^{r}P^r	7	29.4	61.0	107	0.069	0.221	220
I–83	llhhP^{r}P^r	12	41.3	95.3	131	0.041	0.100	144
II–3	llhhP^{r}P^r	14	69.0	127	85	0.121	0.426	252

maximum expansion. At the same time the zone showing anthocyanin also enlarges acropetally closely following behind the hump. Anthocyanin and chlorophyll were never observed to coexist over the same area nor was anthocyanin found in an area which had not yet expanded. The consistent sequential occurrence of bleaching, expansion, and the anthocyanin formation suggested that perhaps the mechanism responsible for anthocyanin synthesis is set in motion by conditions generated in the cell as a consequence of its rapid expansion. These conditions may also be incompatible with the maintenance of chlorophyll in the cells. Curves A and B of figure 1 show the relation of maximum rates of expansion and anthocyanin production.

EFFECTS OF INJURY. Observations on the effects of accidental injury to petals led us to an experiment in which petals were deliberately injured by two needle tears at the time of culturing. Both fresh weight gain and anthocyanin synthesis are stimulated by such treatment (table IV). A time course study on injured petals (fig 2) clearly shows that the periods of most active expansion and coloration occur some 6 to 8 days earlier than in uninjured cultures. To determine the upper limit of stimulation by injury, petals in three parallel sets of cultures were left uninjured, cut in halves, and cut in quarters, respectively. While halving resulted in a doubling of weight and tripling of pigment production further injury by quartering the petals resulted in no further expansion and only a very slight further stimulation of anthocyanin synthesis (table V). In all cases, however, injury stimulated both expansion and coloration.

TABLE V

DEGREE OF INJURY CORRELATED WITH FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS*

TREATMENT	FRESH WT/FLOWER mg	% INCREASE	OD/FLOWER	% INCREASE	OD/g TISSUE	% INCREASE
Petals whole (control)	66.6	...	0.143	...	0.214	...
Petals halved	141.0	112	0.432	198	0.306	43
Petals quartered	138.7	108	0.460	222	0.332	55

* Genotype llhhP^{r}P^r

EFFECTS OF KINETIN. In an attempt to achieve chemical stimulation of expansion, kinetin, reported to have such action on leaf discs by Miller (12), was added to the medium. At the optimum concentration of 0.5 mg/l, kinetin indeed increased the fresh weight of petals (table VI) but its effects on pigmentation were even more pronounced. In general, kinetin greatly increased anthocyanin production. These results are in contrast with Thimann's observations (15) in which he found no stimulation of anthocyanin synthesis by kinetin in his Spirodela system.

TABLE VI

EFFECTS OF KINETIN ON FRESH WEIGHT & ANTHOCYANIN PRODUCTION OF CULTURED PETALS*

KINETIN CONC mg/l	FRESH WT/FLOWER mg	ANTHOCYANIN CONTENT	
		OD/FLOWER	OD/g TISSUE
0.0	83	0.214	0.258
0.5	135	0.571	0.422
1.0	112	0.342	0.306

* Genotype llhhP^{r}P^r

EFFECTS OF MANNITOL & TRIIODOBENZOIC ACID. As it had been found that both chemical and mechanical stimulation of expansion produced similar stimulation of anthocyanin synthesis, an attempt was made to restrict expansion and observe whether or not pigment production would also be inhibited. For this purpose petals were grown in graded concentrations of mannitol to interfere osmotically with water uptake and also in the presence of triiodobenzoic acid (TIBA), a potent antagonist of auxins (7), to suppress expansion by disturbing hormonal controls. The results obtained from the mannitol experiments (table VII) indicate that anthocyanin production is inhibited to a greater degree by higher, osmotically active, concentrations than is petal expansion itself. That no specific toxicity to the pigment producing system is involved may be surmised from the slightly stimulatory effects of the lowest mannitol concentra-

TABLE VII

EFFECTS OF MANNITOL CONCENTRATION ON FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS*

MANNITOL CONC M	FRESH WT/ FLOWER mg	% CHANGE	OD/FLOWER	% CHANGE	OD/g TISSUE	% CHANGE
0.000 (control)	267	...	0.344	...	0.125	...
0.005	277	+4	0.399	+16	0.154	+19
0.010	303	+13	0.384	+12	0.126	0
0.050	211	−21	0.299	−29	0.110	−12
0.100	138	−48	0.084	−76	0.061	−51
0.150	110	−59	0.056	−84	0.050	−60
0.200	105	−61	0.057	−84	0.057	−54
0.250	80	−70	0.034	−90	0.044	−65

* Genotype IIhhP^{r}P^r

tions. Results obtained with TIBA are summarized in table VIII. The most significant information derived from TIBA treatment is not quantitative, however. When cultures were observed after 10 days of incubation the following alteration in the normal sequence of developmental events was noted. Petals in the presence of 5 mg/l TIBA lost their chlorophyll and had expanded considerably without any trace of anthocyanin appearing. Two days later, however, rapid anthocyanin synthesis set in and, as table VIII shows, at the conclusion of the experiment pigment content reached 65 % of the control value. We thus succeeded in creating a time gap between bleaching and expansion on one hand and the appearance of anthocyanins on the other.

TABLE VIII

EFFECT OF TIBA ON FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS*

TIBA CONC mg/l	FRESH WT/ FLOWER mg	ANTHOCYANIN CONTENT	
		OD/ FLOWER	OD/g TISSUE
0.0	154	0.517	0.335
1.0	176	0.500	0.284
5.0	144	0.326	0.226
10.0	PETALS DEAD		
20.0	PETALS DEAD		

* Genotype IIhhP^{r}P^r

EFFECTS OF ETHIONINE. Protein synthesis has frequently been associated with cell expansion (17) but the independence of these two events in some petals has also been demonstrated (11). We therefore grew petals in the presence of ethionine, an amino acid analogue, in an attempt to interfere with protein synthesis and in order to determine whether anthocyanin production and expansion depend on this process. Both anthocyanin production and expansion were sharply reduced (table IX). In an attempt to shed some light on the mechanisms of action of kinetin and ethionine their combined effects on expansion and pigmentation were examined. As table X shows, ethionine completely erases any stimulation by kinetin indicating that both agents may act on the same metabolic sequence. That this sequence involves the synthesis of materials at a specific stage of development is demonstrated by the results of an experiment in which ethionine inhibition was tested on very young as well as more mature petals. Petals from 70 mg buds, although devoid of visible pigment, have apparently partially completed the ethionine sensitive step in the assembly of their anthocyanin producing

TABLE IX

EFFECTS OF ETHIONINE ON FRESH WEIGHT & ANTHOCYANIN CONTENT OF CULTURED PETALS*

ETHIONINE CONC M	FRESH WT/ FLOWER mg	% CHANGE	ANTHOCYANIN CONTENT			
			OD/FLOWER	% CHANGE	OD/g TISSUE	% CHANGE
0.00	81	...	0.22	...	0.272	...
10^{-5}	67	−17	0.24	+09	0.358	+31
6×10^{-5}	34	−38	0.08	−64	0.235	−14
10^{-4}	22	−78	0.06	−73	0.272	...
2×10^{-4}			MOST PETALS DEAD			
4×10^{-4}			MOST PETALS DEAD			

* Genotype IIhhP^{r}P^r

TABLE X

COMBINED EFFECTS OF ETHIONINE & KINETIN ON CULTURED PETALS*

ADDITIONS TO MEDIUM	FRESH WT/ FLOWER	% CHANGE	OD/FLOWER	% CHANGE	OD/g TISSUE	% CHANGE
None	93.7	...	0.238	...	0.254	...
Ethionine 6×10^{-5} M	42.6	−55	0.074	−69	0.174	−31
Kinetin 2.5×10^{-5} M	114.6	+22	0.288	+21	0.252	0
Ethionine + Kinetin	47.4	−49	0.103	−57	0.218	−14

* Genotype llhhP^{r}P^r

mechanism and were less effected by the inhibition. Petals from 30 mg buds, on the other hand, are much more susceptible to such inhibition. In all these experiments we were unable to divorce expansion from anthocyanin production although it is now clear that a time gap may be introduced between the two and that the quantity of pigment produced is not in direct proportion to the degree of expansion.

DISCUSSION

Our results indicate that petals at a bud stage of 25 mg fresh weight and higher possess the complete machinery for the production of anthocyanins. No exogenous substrates are needed beyond inorganic salts, vitamins, and sugar. From their experiments on buckwheat, Underhill et al (18) concluded that quercetin, a flavonol related to cyanidin, may be synthesized in the stem and stored in the leaves. Although a similar relationship between balsam petals and the rest of the plant is not ruled out, isolated petals appear fully competent to produce at least the usual complement and quantity of anthocyanins and flavonols.

The quantitative aspects of pigment production in culture must be tested separately in the dilute pink line and the more intense red and purple lines. In the latter anthocyanin production in culture and on the plant were nearly the same. In the pink line of genotype llhhP^{r}P^r, however, culturing of petals resulted in an eightfold increase in anthocyanin content and thus brought petals to the level of pigmentation normally found in the deep sepals of this line. Anthocyanin production in petals thus appears to operate under a quantitative control mechanism which depends on the continued association of these parts with the rest of the plant. Once petals are removed from such control they may produce higher amounts of pigment. Although both the upper limit of anthocyanin synthesis and the relative pigment content of sepals and petals seems to be governed by the same gene, it follows from our observations that only the former action of this gene is the governing influence residing in the petal cells, while the latter action is exerted somewhere else in the plant. Culturing has the effect of removing this second influence.

The invariable appearance of the 3′,4′-dihydroxy pattern on the B ring of flavonoids found in cultured petals is open to several interpretations two of which will be considered here. It is conceivable that both the higher pigment yield and the presence of additional pigments in cultured petals may be ascribed to the effects of injury incurred in the process of their removal. Indeed, Bopp has recently shown (2) that deliberate injury may induce the appearance of anthocyanins in normally unpigmented structures. The kind of anthocyanin produced was usually the one found in some other portion of the plant. In balsam cyanidin is normally present in the stems of all three and the sepals of two of the three genotypes here investigated. This might lead to the conclusion that, as in Bopp's findings, the appearance of cyanidin in cultured petals is a consequence of injury. Yet when petals are injured in situ no additional anthocyanin is developed and no change in the kind normally present is noted by visual observation.

Developing sepals bear closer resemblance to vegetative leaves than do petals. Sepals remain green and firm longer, expand later and are exposed to the atmosphere and direct sunlight much sooner than petals. In all the genotypes studied, sepals produce the 3′,4′-dihydroxy flavonol quercetin; in two genotypes they produce the corresponding anthocyanin, cyanidin, which is the same pigment found in vegetative structures. Cyanidin is the most widely distributed and also frequently considered to be the primitive and, perhaps, biosynthetically key anthocyanidin (eg. 17). Thus, sepals present, in general, a less specialized morphological and biochemical appearance than petals. Yet, when petals are placed in culture they also produce cyanidin. We suggest that this phenomenon may be ascribed to the partial inhibition of biosynthetic steps which normally convert one of the precursors of this pigment to the other anthocyanins. It is presumably easier to interfere with a highly specialized process than with a more primitive one. Accordingly the interpretation we give to the appearance of cyanidin and quercetin in cultured petals is that the more specialized steps, production of 4′- mono- and 3′,4′,5′-trihydroxy derivatives have been disturbed. Currently available data suggests that the 3′,4′-dihydroxy pattern is introduced

at the level of phenylpropanoid precursors to the B ring. From the order of appearance of pigments in the grape, Ribereau-Gayon (13) considers that cyanidin serves as a precursor of delphinidin and malvidin. Since both anthocyanins and flavonols are similarly affected by culture conditions this would indicate that the hydroxylation pattern is modified in a compound which serves as a precursor to both.

With respect to the developmental physiology of petals as it relates to anthocyanin synthesis, we are still far from a coherent picture. The stimulatory effects of kinetin confirm the view (14, 15) that anthocyanin synthesis is dependent on purine and pyrimidine metabolism. It is gratifying that we were able to enhance rather than restrict pigment production, thus escaping the general criticism leveled against conclusions based on inhibitor studies alone. Nevertheless, the involvement of nitrogen bases in flavonoid synthesis still cannot be tied to a definite mechanism. Thimann and Radner (16) showed that in *Spirodela pyrimidine* effects are mediated by riboflavine metabolism. Strauss, in a recent paper (14) noted the stimulation of anthocyanin synthesis in corn endosperm by nucleotides but could not demonstrate the mediation of riboflavine. Growing petals are neither wholly autotrophic like Spirodela, nor are their cells specialized for the storage of massive amounts of reserves as in corn endosperm. Anthocyanin synthesis in these three experimental objects is superimposed on three markedly different metabolic patterns, and may be most sensitive to experimental intervention at different points along a common course.

Our ethionine results suggest that kinetin may effect expansion and pigmentation by influencing protein synthesis. The close correlation of morphological expansion with biochemical events and the disturbance of this relationship by TIBA may mean that both are triggered by a hormonal mechanism. Anthocyanin production and the disappearance of chlorophyll may be indications of a major shift in metabolism. It is tempting to draw a parallel between this possible shift and the observations of Hotta et al (10) who found that renewed protein synthesis and a change in the kind of ribose nucleic acid formed was closely linked with the onset of a specific morphogenetic stage. The advantage of using a simple pigment as an indicator for a specific morphogenetic step lies in the hope that eventually this may greatly aid in the metabolic characterization of a developmental event.

Summary

I. Immature petals of *Impatiens balsamina* L. continue to expand when they are excised and floated on a simple nutrient medium. Anthocyanin synthesis by such petal tissue was studied.

II. In two intensely colored genotypes (llhhP^{r}P^r & LLhhP^{r}P^r) pigment production per petal was nearly as high in culture as in intact flowers. Anthocyanin content was three times the normal on a fresh weight basis. In a lightly colored genotype (llhhP^{r}P^r) cultured petals produced more than eight times the normal amount of anthocyanin. Pigment content per unit weight was 23 times that of undisturbed petals.

III. Cyanidin appeared as an added anthocyanidin in the petals of all three investigated genotypes when these were placed in culture. Quercetin was found as an added flavonol in two of the genotypes. Peonidin appeared as an added anthocyanidin in one genotype. As a contrast no 3′,4′-dihydroxy flavonoids have been identified in intact petals although they are generally present in the sepals. This may be interpreted as evidence for a return to a more primitive 3′,4′-hydroxy pattern under the influence of culture conditions.

IV. Evidence was found for the sequential appearance of the two major pelargonidin glycosides in two of the genotypes. The order of appearance was: 3-glycoside followed by the 3,5-diglycoside.

V. Morphogenetic steps leading to the expansion and coloration of petals in culture are described. Injury was found to accelerate both expansion and anthocyanin production. Kinetin at 0.5 mg/l had similar effects. When expansion was limited by concentrations of mannitol greater than 0.1 M anthocyanin synthesis was also curtailed. Triiodobenzoic acid at 5.0 mg/l introduced a time gap between expansion and anthocyanin synthesis. Ethionine at 6×10^{-5} M/l reduced both expansion and anthocyanin content. Ethionine completely erased any stimulation by kinetin.

VI. These results are interpreted to point to a close relationship between specific morphogenetic events, such as cellular expansion and the loss of chlorophyll, and the appearance of anthocyanins in petals. Anthocyanins are seen as useful indicators of metabolic conditions accompanying differentiation.

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PYRIMIDINE METABOLISM IN GERMINATING SEEDLINGS[1, 2, 3]

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In the course of an inquiry into the biochemical events associated with germination and dormancy in seeds an attempt was made to assess the turnover or new production of ribonucleic acid and deoxyribonucleic acid in germinating rape seeds. The use of uracil and thymine, both labelled in the 2-position with C^{14} has disclosed that at least in the early stages of germination, these two pyrimidines, the first of which is found in ribonucleic acid and the second in deoxyribonucleic acid, are only sparingly incorporated into polynucleotides although the bases are rapidly decomposed by a sequence of events suggestive of that found in higher animals.

MATERIALS AND METHODS

CHEMICALS USED: Uracil and thymine were obtained from the Nutritional Biochemical Corp., dihydrouracil (DHU)[4] and dihydrothymine (DHT) from the California Corp. for Biochemical Research and were used without further purification. β-Ureidopropionic acid (BUP) and β-ureidoisobutyric acid (BUIB) were made from DHU and DHT, respectively, by alkaline hydrolysis (10). (mp of BUP found, 170–171° C; reported 169–170° C; mp of BUIB, found, 114–116° C, reported, 116–119° C (10). Uracil-2-C^{14} (0.0263 mc/mg) and thymine-2-C^{14} (0.011 mc/mg) were purchased from the New England Nuclear Corp.

GERMINATION OF SEED: The rape seeds (*Brassica napus* L., var. Dwarf Essex) were obtained from the Crawfordsville Seed Co., Crawfordsville, Ind. Germination was carried out in small cylindrical dishes (1 cm high × 1 cm dia.). A disc of Whatman No. 1 filter paper was inserted into each dish. Fifty μliters of a 0.01 % solution of either uracil-2-C^{14} or thymine-2-C^{14} were pipetted into each dish. Five

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[4] The following abbreviations are used throughout: BUIP, β-ureidoisobutyric acid; BUP, β-ureidopropionic acid; DHT, dihydrothymine; DHU, dihydrouracil.

seeds were placed in each dish which was then covered with an inverted dish of a slightly larger diameter. The dishes were supported on a wire gauze in a covered 10 cm petri dish. Sufficient water was present in the petri dish to insure a high relative humidity throughout the germination period. Germinations were carried out in the dark at an average temperature of 25° C. The filter paper disc was omitted from the germination dishes in experiments in which it was desired to determine the unabsorbed radioactivity.

When seeds were germinated under N_2 the small germination dishes were placed into Thunberg tubes, in which they were supported on small vials containing 0.4 ml of water. The Thunberg tubes were alternately evacuated and filled with N_2 to insure exclusion of air.

DETERMINATION OF ACTIVITY INCORPORATED: The seedlings were removed from the germination dishes and thoroughly rinsed with distilled water to wash off any radioactive material adhering to their surfaces. They were then homogenized in ice-cold 80 % ethanol with a Potter-Elvehjem glass homogenizer and made up to known volumes. Aliquots of the suspension were transferred to aluminum planchets containing 0.1 ml of a 0.1 % aqueous agar suspension. The homogenate and agar suspension were thoroughly mixed with a fine wire. To obtain reproducible geometry for radio-isotope measurement the sample was carefully spread over a previously inscribed circle on the planchet and dried under an infra-red lamp. In all the samples counted the weight of the plated material was less than 1 mg/cm^2. Counting was done in a windowless gas-flow counter.

When the amount of unabsorbed activity was to be determined, the rinse water from the seedlings was collected and combined with the rinse water from the germination dishes and aliquots were counted.

CHROMATOGRAPHY: The homogenates of the seedlings were centrifuged at 680 × g for 15 minutes. The supernate was removed by decantation, concentrated in a stream of filtered air, and chromatographed. Chromatography was carried out on Whatman No. 1 paper in cylindrical glass jars (30 cm high × 15 cm dia) in an ascending direction. Development was allowed to proceed for 18 hours using the solvents described by Fink et al (7). The developed chromatograms were dried and scanned for radioactivity using the Forro scanning device in conjunction with a recording rate-meter. Pyrimidines, dihydropyrimidines and β-ureido acids were detected by the procedures of Fink et al (9). Nucleic acid hydrolyzates were neutralized with solid K_2CO_3 and after removing the $KClO_4$ by centrifugation, were concentrated and chromatographed using solvent system I of Kirby (15).

ISOLATION OF NUCLEIC ACIDS FROM SEEDLING EXTRACTS: The homogenates of the seedlings were fractionated according to the method of Ogur and Rosen (16). The combined nucleic acids were extracted and hydrolyzed to their constituent bases as described by Basler and Commoner (3). Preliminary experiments indicated virtually complete recovery of nucleic acids from the tissue by this procedure.

RESULTS

When rape seeds were germinated in solutions of uracil-2-C^{14} or thymine-2-C^{14}, radioactivity appeared in the seedlings reaching a maximum, and then decreasing. Some typical results are shown in figures 1 and 2. That the later decrease was not caused by loss of pyrimidine or radioactive metabolites by leaching is clear from the fact that radioactivity remaining in the germination vessel also declined. It is noteworthy that an appreciable amount of radioactivity from both thymine and uracil was incorporated into the seeds after only 6 hours of contact; the primary shoot was not observed to emerge until after 12 hours of germination. From these experiments and others it is apparent that absorption of the radioactive pyrimidines can occur prior to breaking the testa. With increasing time the activity present in the unabsorbed fraction decreased until it had virtually disappeared. Ultimately about 90 % or more of the original activity was lost; the remainder was found in the seedlings.

Parallel experiments carried out in the presence of NaOH to trap the CO_2 showed that the dissipated radioactivity was being evolved as $C^{14}O_2$. Thus when ten seeds were germinated for 48 hours in 0.2 ml of a 0.01 % solution of uracil-2-C^{14}, 45 % of the original activity was recovered as carbonate. In a similar experiment with 0.2 ml of a 0.015 % solution of thymine-2-C^{14}, 44 % of the activity was recovered.

Employing solvent 2 (7), chromatography of the extracts obtained from seedlings grown, for various periods in uracil-2-C^{14}, resulted in the appearance of two radioactive regions (fig 3). One of the regions coincided with BUP, while the other corresponded with both uracil and DHU which are not well resolved by this solvent. However, by using solvents 1 and 6 (7), which gives an excellent resolution of these substances, it was found that DHU-C^{14} as well as unreacted uracil-C^{14} were present in seedlings germinated for 12 hours (fig 4). Similarly with seeds which were germinated in thymine-2-C^{14}, solvent 2 (8) resolved the radioactive substance into two areas, thymine-DHT and BUIB-C^{14} (fig 5). Application of solvents 1 and 6 (7) showed the thymine-DHT region contained both thymine-C^{14} and DHT-C^{14} when the extract was obtained from seedlings germinated in thymine-2-C^{14} for 12 hours in air (fig 6).

Although large fractions of the bases were catabolized, some incorporation into nucleic acid occurred. Thus when 15 seedlings obtained by germination on H_2O for 72 hours were transferred to 0.75 ml of a 0.01 % aqueous solution of uracil-2-C^{14} for 24 hours, approximately one percent of the radioactivity was found in nucleic acid. This represented 32 % of the total activity in the homogenate. Radio-

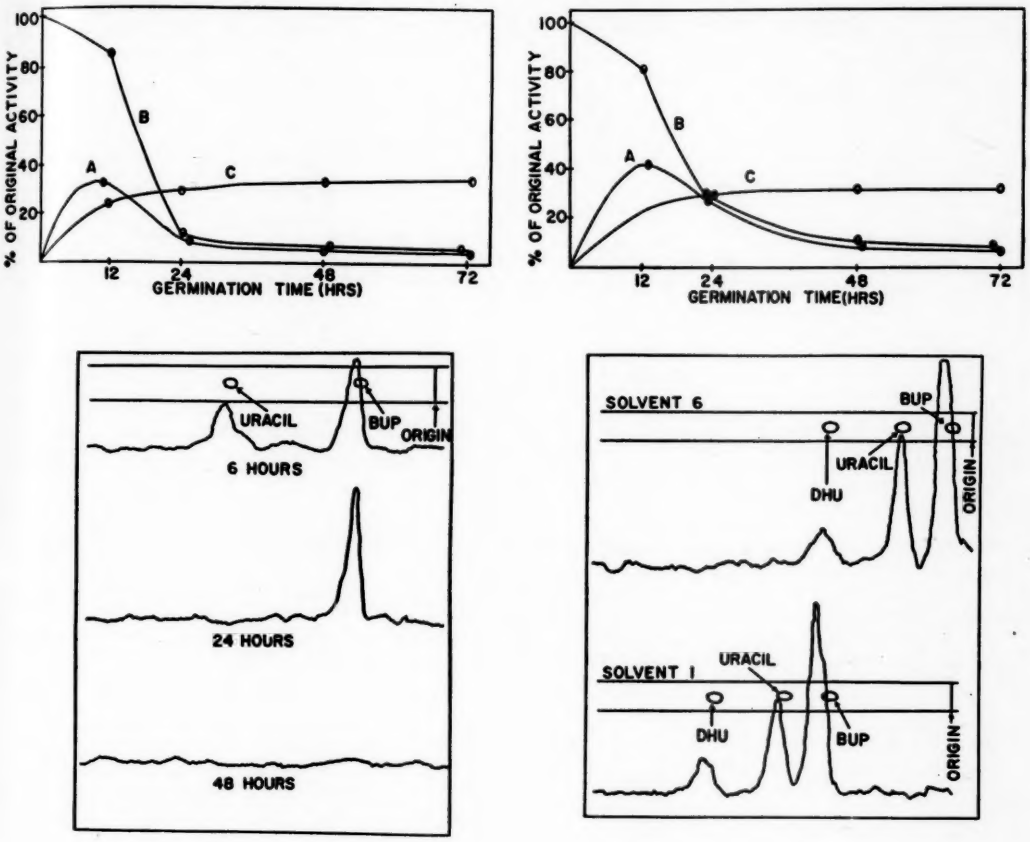
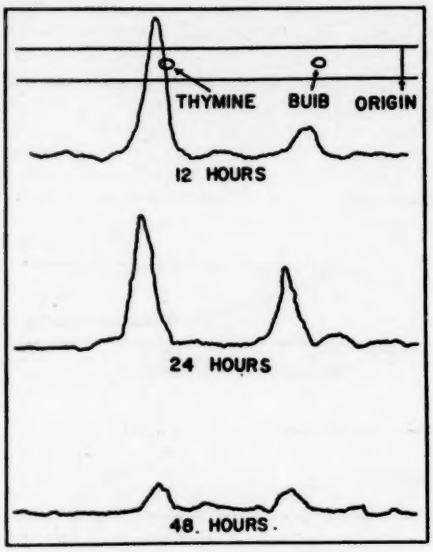


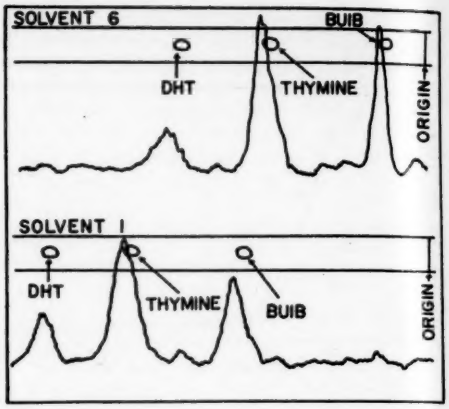
FIG. 1 (*upper left*). Time course of incorporation of radioactivity from uracil-2-C^{14} into germinating rape seeds. Radioactivities are expressed as percent of the total activity originally added to the germination vessel. A: Radioactivity found in seedlings (germination in air). B: Radioactivity remaining in germination vessel (germination in air). C: Radioactivity found in seedlings (germination in N_2).

FIG. 2 (*upper right*). Time course of incorporation of radioactivity from thymine-2-C^{14} into germinating rape seeds. Radioactivities are expressed as percent of the total activity added to the germination vessel. A: Radioactivity found in seedlings (germination in air). B: Radioactivity remaining in germination vessel (germination in air). C: Radioactivity found in seedlings (germination in N_2).

FIG. 3 (*lower left*). Radio-isotope scannings of chromatograms of supernates from homogenates obtained from seeds germinated for 6, 24, and 48 hours in uracil-2-C^{14}; solvent 2 (7).

FIG. 4 (*lower right*). Radio-isotope scanning of chromatograms of supernates of homogenates from seedlings germinated for 12 hours in uracil-2-C^{14}. *Upper figure,* solvent 6; *lower figure,* solvent 1, (7).





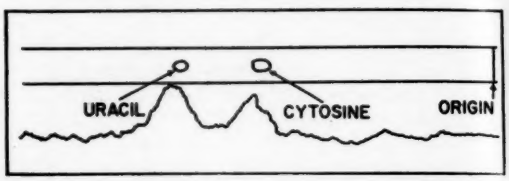


FIG. 5 (*upper left*). Radio-isotope scanning of chromatograms of supernates of homogenates from seedlings germinated for 12, 14, and 48 hours in thymine-2-C^{14}; solvent 2 (7).

FIG. 6 (*upper right*). Radio-isotope scanning of chromatograms of supernates of homogenates from seedlings germinated for 24 hours in thymine-2-C^{14}; solvent 1 and 6 (7).

FIG. 7 (*lower right*). Radio-isotope scanning of chromatograms of the hydrolyzed nucleic acids from seedlings germinated for 72 hours in water followed by 24 hours in uracil-2-C^{14}; solvent 1 (15).

active uracil and radioactive cytosine were identified among the hydrolytic products (fig 7). The formation of cytosine from uracil at the nucleotide level is well known to occur in other organisms. The ratio of counts in uracil to cytosine was approximately 1:1 indicating a substantial conversion. In these experiments no effort was made to separate the DNA from the RNA. When 72 hour-old seedlings were transferred to a 0.01 % solution of thymine-2-C^{14} for 24 hours radioactive nucleic acid was obtained but the low level of activity did not permit the identification of radioactive bases present in the hydrolytic products after chromatography.

DISCUSSION

The production of $C^{14}O_2$ β-ureidopropionic acid and dihydrouracil from uracil-2-C^{14} by germinating rape seeds strongly suggests the following pathway is involved:

Uracil → dihydrouracil → β-ureidopropionate → β-alanine + CO_2 + NH_3

Similarly the formation of $C^{14}O_2$, dihydrothymine and β-ureidoisobutyrate from thymine-2-C^{14} suggests operation of the following catabolic scheme:

Thymine → dihydrothymine → β-ureidoisobutyrate → β-aminoisobutyrate + CO_2 + NH_3

Apparently the catabolic pathways in germinating rape seeds are not unlike those reported in higher animals and in some bacteria (4, 5, 8, 9, 11, 12, 13, 19). In the experiments described here, one would not expect to find labeled β-alanine or β-aminoisobutyrate owing to the loss of the C^{14}-labeled C-2 atom as CO_2. Attempts to demonstrate the formation of these compounds from non-radioactive uracil and thymine have not been successful apparently because of the relatively low enzyme activities in the seedlings.

The presence of these catabolic sequences is consistent with the established decrease of RNA phosphorus in seeds during germination (17).

These reactions are the only ones known to occur in higher plants which can account for the formation of β-aminoisobutyrate which has been found in iris (2). β-Alanine in higher plants (14) could also arise from these reactions, although both squash (18) and various leguminous seeds (1) have been reported to catalyze the evolution of CO_2 from L-aspartate. However, it remains to be shown that in these cases the reaction is caused by an aspartic acid decarboxylase.

Note that degradation of uracil and thymine involve only a hydrogenation step followed by hydrolytic steps. It is therefore interesting that the exclusion of oxygen from germinating seeds represses the catabolism of these substrates. This behavior

may be a secondary effect resulting from the overall inhibition of germination brought about by anaerobiosis and the consequent inhibition of developmental changes such as formation of new enzymes or coenzymes, alterations in subcellular structure, or permeability changes needed to permit the substrate to come in contact with the enzyme.

As far as we know the individual enzymes concerned with each of the steps shown here have not been demonstrated in higher plants. The isolation of the enzymic reactions is under way in our laboratory. Wallach and Grisolia have purified a hydrase from rat liver which attacks dihydrothymine, dihydrouracil, and hydantoin (19). They have suggested that the hydantoin peptidase discovered by Eadie et al (6) some years ago in seeds may also act on the dihydropyrimidines. In a study to be reported later we have partially purified a preparation of dihydrouracil hydrase from pea seeds which also opens the rings of dihydrothymine and hydantoin, but the results thus far do not permit a decision as to whether or not these activities reside in a single enzyme.

The ability of the seedlings to incorporate uracil into nucleic acid suggests, by analogy with other organisms, the action of a nucleoside phosphorylase to produce a nucleoside which is then converted by the action of a nucleoside kinase to a 5′-nucleotide. This in turn would be converted to the di- or triphosphate. It is interesting, but not unexpected, that uracil can serve as a precursor of cytosine.

Summary

I. Germinating rape seeds rapidly absorb and decompose added uracil and thymine.

II. Among the catabolic products, arising from these bases in addition to CO_2, are dihydrouracil and β-ureidopropionic acid from uracil, and dihydrothymine and β-ureidoisobutyric acid from thymine.

III. In addition to these catabolic reactions, incorporation of the pyrimidines into nucleic acids can be shown.

IV. When uracil is incorporated into nucleic acid it gives rise to both uracil and cytosine.

Acknowledgments

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EFFECT OF CATIONS ON GERMINATION & GERM TUBE DEVELOPMENT OF PUCCINIA CORONATA UREDOSPORES[1,2]

H. MELVIN COUEY[3] & FREDERICK G. SMITH

Previous work in this laboratory (20) showed that germ tubes of *Puccinia coronata* uredospores would form structures in vitro resembling those found in infected host plants (18). This so-called vesicle formation occurred on Difco gelatin in the pH range of 6.2 to 6.9 on addition of 14 to 22 $\times$ 10^{-5} M Zn. Under these conditions germ tube length was reduced, and in some cases the percentage germination was lowered. Other metals, including Cd, Cu, Fe, Mg, Ca, Mo, Co, and Mn, did not replace Zn.

Several preliminary observations in Sharp's work (19) and in the present study indicated gelatin contained substances affecting the action of Zn on germ tube growth and differentiation. A. The extent of vesicle formation varied with different gelatin samples. B. The concentration of Zn required increased with the gelatin concentration. C. A water extract of gelatin with added Zn stimulated vesicle formation on an agar substrate at pH 6.3 to 6.5 D. When gelatin was purified by water extraction, adding Zn failed to induce vesicle formation, and Zn inhibited germination at much lower concentrations. This paper reports further study of the action of Zn and gelatin under better defined conditions and presents evidence that both germination and vesicle formation are subject to cation effects.

Materials & Methods

Uredospores were mixed race collections of *Puccinia coronata* Corda var. avenae F. and L. from both the field and greenhouse. The germination substrate ordinarily was 4 ml of 2% Difco gelatin in 6 cm petri plates. Spores were applied from a small cyclone duster (22). With liquid substrates, spores were added to 2 ml volumes in 5 ml beakers by transferring loops of spores floating on water. The germination vessels were covered loosely or placed in a moist chamber and incubated at 20 to 21° C in the dark. Under these conditions, no evidence was observed of the self inhibition of germination reported for *P. graminis* tritici uredospores (2, 6).

Germination counts were made after about 12 hours and vesicle formation counts after 36 to 40 hours. A spore was counted as germinated if the germ tube length was greater than the spore diameter. The formation of a spindle-shaped substomatal vesicle was the minimum requirement for counting as vesicle formation since a distinct appressorium was sometimes missing at high Zn concentrations. Five fields of 35 to 50 spores each were examined per plate. Ordinarily, a single plate for each treatment was used, and the experiments were repeated several times. Statistical analysis was not regularly applied, but previous experience (21) and several analyses of replicate experiments indicated that a difference of about 10 to 12 in percentage germination was significant at the 5% level.

Only samples of Difco gelatin which supported vesicle formation were used. Ion-exchange purification was carried out first with a carboxylate resin (Amberlite IRC–50) in the Na form and later with a mixed bed column of a strong acid and strong base resins (Amberlite IR–120 and IRA–400) in the H and OH forms, respectively. Both systems maintained a pH near enough to neutrality to avoid gelatin hydrolysis. The columns were held at 40 to 45° C to prevent solidification of the gelatin solutions. Flow rates and quantities of gelatin were kept well below the exchange capacity of the columns. The mixed bed system was more efficient in cation removal, and the effluent gelatin was close to the isoelectric point. Preparations from the carboxylate column will be referred to as "cation-free" gelatin though the gelatin cations actually were replaced by Na ions. The preparation from the mixed bed column is called deionized gelatin.

Results

Effect of Cation Exchange Treatment of Gelatin on Germination and Vesicle Formation: The first evidence that other cations affected the action of Zn was observed with cation-free gelatin as substrate. When the native gelatin cations were replaced by Na ions, Zn was more toxic and by itself no longer would induce vesicle formation (table I and fig 1). The percentage germination in the absence of added Zn also was reduced on the treated gelatin (fig 1). Adding Ca or Mg reduced the toxicity of Zn and restored its ability to promote vesicle formation (table I). A mixture of the two cations usually was more effective than either one alone. Since the level of vesicle formation under these conditions was roughly equivalent to that observed on the corresponding whole gelatin, it appeared that cations other than Ca and Mg were not required.

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[2] Journal Paper No. 3880 of Iowa Agricultural and Home Economics Experiment Station, Ames. Project No. 1258. These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Iowa State University, NR 103-109.

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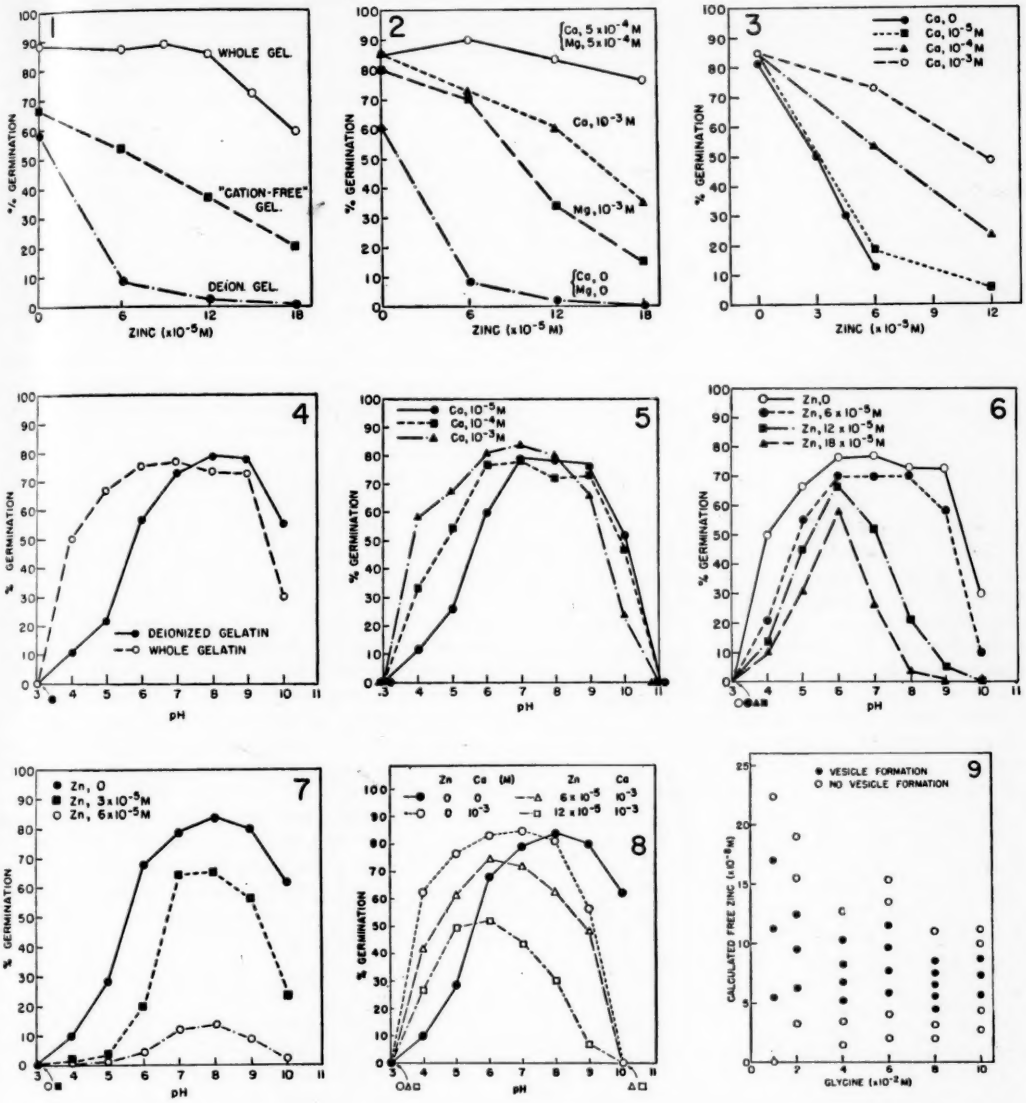

FIG. 1. Effect of Zn concentration on germination at pH 6.5 on whole, cation-free, and deionized gelatins.
FIG. 2. Effect of Ca and Mg on Zn inhibition of germination at pH 6.5 on deionized gelatin.
FIG. 3. Relation of Ca concentration to Zn inhibition of germination at pH 7 on deionized gelatin.
FIG. 4. Germination on whole gelatin and deionized gelatins as a function of pH.
FIG. 5. Germination on deionized gelatin as a function of pH and Ca concentration.
FIG. 6. Germination on whole gelatin as a function of pH and Zn concentration.
FIG. 7. Germination on deionized gelatin as a function of pH and Zn concentration.
FIG. 8. Germination on deionized gelatin as a function of pH and Zn and Ca concentration.
FIG. 9. Vesicle formation on glycine solutions as a function of glycine and calculated free Zn concentration.

TABLE I

PERCENTAGE GERMINATION & VESICLE FORMATION ON CATION-FREE GELATIN CONTAINING Zn AS FUNCTION OF Ca & Mg CONCENTRATION

	Mg ($\times 10^{-4}$ M)	Ca ($\times 10^{-4}$ M) 0	2.5	5.0	7.5	10
Germination	0	27	33	31	41	40
	2.5	58	57	62	64	62
	5.0	54	70	66	59	68
	7.5	49	65	71	52	74
	10.0	35	61	63	58	45
Vesicle formation	0	0	0	1	0	0
	2.5	1	1	4	10	4
	5.0	0	3	11	4	2
	7.5	1	3	9	6	3
	10.0	0	7	12	3	7

Zn = 18×10^{-5} M, pH = 6.5

EFFECT OF CATION INTERACTION IN GERMINATION ON DEIONIZED GELATIN: With deionized gelatin as substrate, Zn was even more toxic than with cation-free gelatin (fig 1). This was attributed to the more complete removal of antagonistic ions. Adding sufficient Ca or Mg reduced the toxic action of Zn, and again an equimolar mixture of the two was more effective than either one alone (fig 2). Adding Ca or Mg also raised the percentage germination in the absence of added Zn at pH 6.5 (fig 2) but not at pH 7.0 (fig 3). This difference suggested that H ion toxicity occurred at the lower pH in absence of antagonistic cations. It appeared that the interaction of Ca and Zn could be studied at pH 7 without the complication of H ion toxicity. In the representative experiment in figure 3 about a 100-fold increase in Ca was required to balance the effect of a 4-fold increase in Zn, and 10^{-3} M Ca did not fully reverse the effect of only 6×10^{-5} M Zn.

In the early stages of this work the pH used routinely was 6.4 to 6.5, the level found optimum for vesicle formation by Sharp and Smith (20). However, it became clear that deionization of the gelatin changed the ionic environment to such an extent that it was necessary to reexamine the influence of pH on both germination and vesicle formation. No other buffer was added since the gelatin itself provided adequate buffering over the desired range. On whole gelatin some germination occurred from pH 4 to 10 with maximum percentage from about pH 5 to 9. With deionized gelatin the acid branch of the pH response curve was shifted to higher pH values, and a narrower range of maximum germination from about pH 7 to 9 was obtained (fig 4). This shift in pH response explained the depressed germination due to deionization observed earlier (fig 1) since the pH of 6.5 used at that time is on the shoulder of the curve. Since this change in pH response apparently was due to removal of soluble cations from the gelatin, the effect of adding Ca or Mg was investigated. These cations shifted the acid branch of the curve back to lower pH's (fig 5). The effect increased from 10^{-5} M Ca (indistinguishable from the control, fig 4) to about 10^{-3} M Ca. Similar results were obtained with Mg. The high level of Ca (fig 5) also inhibited germination in the alkaline region, causing a difference from the control curve similar to that between curves on deionized and whole gelatin. However, this effect of Ca was not as consistent or as large as that in the acid region.

Next, the effect of Zn on the pH-germination curves was investigated. It proved to be toxic throughout the pH range with both whole and deionized gelatin (fig 6 & 7). With whole gelatin (fig 6) Zn was relatively more toxic at higher pH's, so the pH optimum for partially inhibited germination was about 6. With deionized gelatin (fig 7) Zn was much more toxic than with whole gelatin over the broad pH range of 4 to 9 as well as at the pH of 6.5 used earlier. The region of maximum germination (pH 7–9) was the same both with and without Zn, indicating that Zn toxicity was not greatly influenced by H ion concentration. Adding Ca to deionized gelatin containing Zn (fig 8) shifted the pH range of maximum germination back to the acid side and decreased the toxicity of Zn (cf. fig 7). These effects of added Ca were similar, though not identical, to the differences in pH-germination curves for whole and deionized gelatin containing toxic levels of Zn. It was concluded that Zn toxicity was influenced more by metallic cation impurities in the gelatin than by H ions.

Although the influence of other cations on H and Zn toxicity was not studied extensively, Mn and Ba at 10^{-3} M gave effects like Ca and Mg, but K and Na did not. It is possible, therefore, that a number of polyvalent cations may influence the action of H and Zn on uredospore germination on gelatin.

EFFECT OF CATIONS ON VESICLE FORMATION WITH DEIONIZED GELATIN: The failure of Zn to induce vesicle formation in the pH range 6.2 to 6.9 (20) after cation removal from gelatin in the present work prompted a reexamination of the effect of H ion concentration on germ tube differentiation. A few germinating uredospores (1–2 %) formed vesicles on the Zn-deionized gelatin substrate at pH 8 and 9 but not at lower pH's. Adding Ca, Mg, or mixtures of the two increased vesicle formation at pH 8 to about the level found on whole gelatin. These experiments also indicated that the required concentration of added cation was dependent on the Zn concentration. The most consistent results were achieved with 10^{-4} M Ca and 4 to 18 $\times 10^{-5}$ M Zn. Table II summarizes a typical experiment comparing vesicle formation as a function of H ion concentration on whole and deionized gelatin. The pH range permitting vesicle formation on this whole gelatin sample was 6.5 to 7.5

TABLE II

PERCENTAGE VESICLE FORMATION AS FUNCTION OF pH & Zn CONCENTRATION ON WHOLE GELATIN OR DEIONIZED GELATIN WITH ADDED Ca

SUBSTRATE	Zn ($\times 10^{-5}$ M)	pH 6.0	6.5	7.0	7.5	8.0	8.5
Whole gelatin	0	0	0	0	0	0	0
	4	0	1	0	0	0	0
	8	0	4	4	1	0	0
	12	2	0	16	9	0	0
	18	1	6	25	*	*	*
Deionized gelatin + 10^{-4} M Ca	0	0	0	0	0	0	0
	4	0	0	0	0	1	3
	8	0	0	1	11	17	34
	12	0	0	0	25	31	*
	18	0	0	*	*	*	*

Percentage vesicle formation on basis of germinated spores
* Indicates conditions permitting less than 20 % germination

while the range after deionization and addition of Ca was 7.5 to 8.5. In other experiments it extended to pH 9. As a whole, the results showed that the pH range for vesicle formation on Ca-Zn-deionized gelatin was wider and higher than on whole gelatin.

VESICLE FORMATION ON AQUEOUS SUBSTRATES: When it was clear that the pH range for vesicle formation varied with the ionic conditions, aqueous substrates containing amino acids and various cations were studied. Considerable vesicle formation was found with 0.01 M glycine and 1 to 4 $\times$ 10^{-5} M Zn at pH 8. Although accurate counts of percentage response were difficult on the liquid substrates, a number of estimates indicated that about 20 % of the germinating uredospores were undergoing typical germ tube differention. No vesicle formation occurred without addition of both Zn and glycine, but Ca or Mg was not required. As on deionized gelatin, the pH optimum was near 8. This pH was used in subsequent experiments.

To clarify the role of the glycine, the effect of the amino acid concentration on the Zn level required for vesicle formation was examined. The data in table III show that increasing the glycine concentration raised the level of Zn required. Similar results were obtained with alanine and valine. The free Zn concentration was calculated according to equations given by Rabin and Crook (16) using affinity constants determined by Maley and Mellor (15). A graph of vesicle formation as a function of free Zn and glycine concentrations is shown in figure 9. Vesicle formation was essentially independent of amino acid concentration from 0.01 to 0.1 M. Graphs for alanine and valine were similar. It was concluded the amino acids were acting primarily, if not exclusively, as Zn buffers. In this way a reservoir of Zn could be maintained without reaching the toxic level of free Zn.

The lack of a Ca or Mg requirement for vesicle formation on amino acid solutions as compared with deionized gelatin posed an interesting question that seemed to involve Zn toxicity and factors affecting it. At pH 8 on 0.001 M tris buffer, Zn inhibited germination throughout the concentration range (10^{-5} M or greater of total Zn) that induced vesicle formation on amino acid solutions. However, if Ca or Mg was added to antagonize Zn toxicity, some vesicle formation occurred under the following conditions: A. 10^{-4} M Ca + 10^{-5} M Zn, B. 10^{-4} to 10^{-3} M Mg + 1 to 2 $\times$ 10^{-5} M Zn, or C. Ca and

TABLE III

VESICLE FORMATION AS FUNCTION OF Zn & GLYCINE CONCENTRATIONS AT pH 8

GLYCINE M	Zn ($\times 10^{-5}$ M) 0	1	2	3	4	6	8	10	12	16	20	24	30	40	50	60	70	80	90	100	120	140	160
0.01	0	+	+	+	±	0																	
0.02	0		0		+	+	+	±	0														
0.04	0				0		0		+	+	+	+	0										
0.06	0							0			0		+	+	+	+	0	0					
0.08	0										0		0	+	+	+	+	+		0			
0.10	0															0	0	+	+	+	+	0	0

+ Indicates vesicle formation, 0 no vesicle formation, ± variable results

Mg at 5×10^{-4} M each + 1 to 4×10^{-5} M Zn. There was some indication that vesicle formation was dependent on the relative concentrations of Zn and the protective cation (Ca or Mg), but this relationship requires further study. Similar results were obtained with low concentrations of amino acids at pH 8. For example, on 0.001 M glycine addition of protective cations reduced Zn toxicity, and vesicle formation occurred under the following conditions: A. 10^{-3} M Mg + 3 to 4×10^{-5} M Zn and B. Ca and Mg at 5×10^{-4} M each + 4×10^{-5} M Zn. These experiments suggested that a certain minimum level of Zn was required for vesicle formation, but in the absence of antagonistic cations or binding agents this concentration completely inhibited germination. This conclusion led to the idea that an amino acid might substitute for Ca or Mg in permitting vesicle formation on deionized gelatin. However, no success was achieved in several attempts with various levels of glycine and Zn.

Discussion

This study presents evidence that the growth and differentiation of *P. coronata* germ tubes are sensitive to the interaction of Zn, H, and Ca or Mg ions. With deionized gelatin as substrate it could be demonstrated that divalent cations such as Ca and Mg reduce Zn inhibition of germination. Such antagonistic action of Ca and Mg toward toxic cations like Zn has been reported for a number of microorganisms including several fungi (1, 11, 12, 14) though apparently not previously for the rust fungi. A significant interaction between H ions and Ca and Mg ions also was established on deionized gelatin. This type of cation interaction has not been widely observed with fungi perhaps because it is obscured by cations in the nutrients or substrates commonly used. However, similar shifts by alkaline earth cations in the pH-respiration curve of yeast (17) and the pH-growth curve of a fungus colony (13) have been reported.

What do the observed pH effects suggest about the nature of the uredospore germination process? From one point of view, the shift in the acid branch of the curve toward lower pH values may be regarded as antagonism of H ion toxicity by Ca and Mg. The roughly bell-shaped nature of these curves indicates that the germination process is controlled by dissociation of acidic groups at the cell surface. For germination to occur, some groups with ionization constants between pH 5 and 6 must be in the dissociated form while other groups with constants around pH 10 must be in the undissociated form. To explain the shift in the acid branch of the curve with increasing concentrations of Ca or Mg, one may assume that binding these cations causes an increase in the acidity of the low pH dissociating groups as has been shown with proteins (10). The validity of this viewpoint must be tested further. More extensive data are needed, particularly with substrates other than gelatin, and more direct methods must be employed to establish whether these cation effects are actually equilibrium phenomena. If this mechanism of cation interaction in the germination process can be substantiated, it would suggest that bound Ca, Mg, or similar cations may be required for growth perhaps as constituents of metallo-enzymes as MacLeod and Snell (14) have proposed in similar work on Lactobacilli. Hydrogen and Zn ions then may act by displacing and controlling the activity of the native cations.

This work also has demonstrated that the ability of Zn to induce vesicle formation by germinating uredospores of *P. coronata* is greatly influenced by other constituents of the substrate. On whole gelatin (20) this phenomenon was restricted to the pH range of 6.2 to 6.9. When the gelatin was purified to remove ionic material, Zn alone was not effective. On addition of Ca or Mg and lowering of the H ion concentration to a pH of about 8, vesicle formation was restored. This higher pH requirement also was observed with amino acid solutions and in some unreported experiments with agar as substrate. Hydrogen ions as well as the divalent cations seemed to affect the activity of the Zn in inducing vesicle formation. Experiments with amino acid solutions provided further evidence that suitable control of Zn activity would result in germ tube differentiation. On these substrates no addition of Ca or Mg was necessary. The amino acids appeared to maintain the appropriate free Zn concentration by acting as Zn buffers. With a low amino acid concentration or with tris buffer, however, adding Ca or Mg was necessary to reduce the toxicity of Zn sufficiently to permit vesicle formation. It may be concluded that Zn alone is capable of inducing vesicle formation in germinating uredospores of *P. coronata* if its activity is properly regulated.

Any consideration of the mechanism of Zn action in inducing germ tube differentiation must recognize the concomitant reduction in germ tube growth and spore germination. This suggests that other factors inhibiting germ tube growth might induce differentiation. This possibility has not been tested extensively, but experience so far indicates that it is unlikely. The specificity of Zn remains to be established, however, since other germ tube growth inhibitors have not been studied over as wide a range of conditions as has Zn.

Some remarkable reports have appeared on factors affecting germination and germ tube differentiation of *P. graminis* tritici. Allen (3) and French, Massey, and Weintraub (7) have shown that a distillate from a uredospore extract would retard germ tube growth and induce vesicle formation. The latter group (8) also have isolated pelargonaldehyde from the distillate and reported that it is active. In addition, Fuchs and Gaertner (9) have observed marked stimulation of vesicle formation by cysteine and glutathione in the presence of iron compounds on a mineral-salts-glucose-silica gel substrate. Finally, Emge (5) has shown that vesicle formation can be

induced on various artificial substrates by the proper sequence of light and temperature conditions. These recent reports along with Dickinson's earlier germination studies (4) on artificial membranes and the work in this laboratory would indicate that a variety of factors can induce rust germ tube differentiation. It remains to be seen how much species differ in response to these various treatments and whether or not any common mechanism is involved.

Summary

Evidence is presented that the cation composition of artificial substrates influences the germination and germ tube differentiation of uredospores of *Puccinia coronata* Corda var. avenae F. and L. Zinc and H ion toxicities were antagonized by added Ca and Mg. Hydrogen ions and the alkaline earth cations affected the ability of Zn to induce differentiation of infection structures. However, Zn alone was effective if its activity was properly controlled by amino acids acting as metal buffers. It is suggested that the observed cation interactions in germ tube growth and differentiation may involve displacement of native cations.

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CHANGING RESPIRATORY PATHWAYS IN POTATO TUBER SLICES[1,2]

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It has long been known that freshly cut pieces of tissue from certain tubers and fleshy roots immediately respire considerably more rapidly per unit mass than tissue in the intact organ, and further that sufficiently thin slices of such tissue exhibit an additional respiratory rise during one to several days of incubation. These phenomena have been observed in slices of potato (5, 19, 22, 23, 24, 25), carrot (20, 27), beet (3, 21, 26), Jerusalem artichoke (26), and chicory root (11). Laties (9) made an analysis of some of the factors involved in the secondary respiratory rise.

The slow oxygen uptake of freshly cut potato slices is inhibited by cyanide and carbon monoxide, but the more rapid respiration which develops during aging is quite resistant to these agents (6, 15). It seems likely that a change in electron transport pathways occurs (6). Changes in respiratory carbon pathways also are indicated. The results of early work with chicory root suggest that an increase in tricarboxylic acid cycle (TCAC) activity occurs during aging. The initial respiration is malonate resistant, whereas the respiration induced by aging is malonate sensitive (11). Recent work by Laties (pers communic) has shown that potato slices undergo a similar increase in sensitivity to malonate during aging. The malonate resistance of fresh tissue raises the possibility that the respiration initially is largely mediated by systems other than the TCAC. We have concentrated on this carbon pathway aspect of the problem.

The plan was to develop techniques permitting use of C^{14} labeled sugars as tracers of respiratory pathways in slowly respiring tissues, by extracting and isolating labeled metabolic intermediates arising therefrom; and to employ glucose specifically labeled on the first and sixth carbon atoms, respectively, as a test for respiratory systems, such as the pentose phosphate pathway (PPP), which can discriminate between these two atoms. We concentrated primarily upon fresh tissue because of its greater physiological interest, and because experience with fresh tissue might serve to elucidate other problems concerning dormancy and development.

MATERIALS & METHODS

TISSUE SOURCE & PREPARATION: All potato (*Solanum tuberosum* L.) tubers used were of the Russet variety. Various lots were used during 18 months of preliminary experiments, but in all experiments of our final series we utilized tissue from a single lot of tubers from the crop harvested in the Shafter, Cal., area in early June, 1959. The tubers were stored at 10° ± 1° C until used during the next 3 months.

Tissue for the several treatments of an experiment was prepared as one lot. From the central part of each of 12 tubers, 24 slices 1 mm thick and 8.8 mm in diameter were cut with a cork borer and a table microtome. The slices, cut directly into water at 0° C, were rinsed with four more changes of ice water. Samples were counted onto bibulous paper saturated with ice water and then placed into flasks for treatment. These slices are referred to as fresh. Temperatures near 0° C inhibit development of the metabolic changes characteristic of aging. Thus the physiological freshness of the slices was insured by keeping them at 0° C until the experimental zero time.

Aged slices were prepared by incubating fresh slices in 10^{-4} M calcium sulfate solution on a rotary motion shaking table (70 cycles/min) at room temperature (26°–29° C) in the dark for 24 to 36 hours. The solution (25 ml/g of tissue) was changed hourly for 3 hours, then less frequently for at least ten changes in 24 hours.

Before incubating with radioactive sugar, fresh tissue samples were preincubated at 0° C for an hour either in 10^{-4} M calcium sulfate solution or in the test solutions. The solutions were replaced with fresh aliquots after 30 minutes. Preincubation served as a further washing period to remove contents of broken cells from the cut surfaces and, in the case of malonate treatments, to allow malonate permeation prior to presenting glucose.

The test solutions were of two formulations, for controls (C), and for malonate treatments (M). Both contained 1×10^{-4} M calcium sulfate. The M solution also contained 5×10^{-2} M malonic acid and was adjusted to pH 5.0 with potassium hydroxide. Malonic acid and potassium hydroxide at a hundredth the M concentration were added to C. This was sufficient to hold the pH near 5.0, but had no respiratory effect. Potassium chloride was added to C to

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balance the potassium ion concentration of M.

A Warburg apparatus set at 28° C was used for manometric experiments. Each flask contained 3.5 ml of C or M solution, ten tissue slices, and 0.2 ml of 10 % NaOH solution and a fluted paper wick in a sidearm. All treatments were run in triplicate.

INCUBATION WITH RADIOACTIVE GLUCOSE: Glucose randomly labeled with C^{14} and having a specific activity of 45.9 microcuries per micromole ($\mu C/\mu M$) was obtained from Isotopes Specialties Co., Inc., Burbank, Cal. Several lots of D-glucose-1-C^{14} and D-glucose-6-C^{14}, having specific activities ranging from 2.19 to 7.7 $\mu C/\mu M$, were obtained from Nuclear-Chicago Corp., Chicago, Ill. Specific activities of all labeled sugars used in any one series of experiments were adjusted to that of the lowest. Labeled sugars were stored dry and cold in small sublots. Prior to each experiment aliquots were dissolved in water and diluted to the desired specific activity, then transferred to the experimental flasks and evaporated to dryness under nitrogen. The dry sugars were redissolved in the test solutions. This procedure eliminates errors due to spontaneous $C^{14}O_2$ evolution which might otherwise occur.

All tissue incubations with labeled substrates were done at room temperature (26°–29° C) in 125 ml flasks on a rotary shaking table (70 cycles/min). At zero time 40-slice samples (approx 2.2 g fresh wt or 0.4 g dry wt) which had been preincubated in nonradioactive test solutions, were added to the experimental flasks containing the labeled sugars dissolved in 7.5 ml of C or M solution. The flasks then were immediately closed with rubber stoppers equipped with hooks and paper loops (Whatman no. 1, 1 × 8 cm strips) saturated with 10 % sodium hydroxide solution.

At the end of each 10.0 minute period the stoppers were quickly replaced with others bearing alkali-saturated papers. Exposed papers were dropped into tubes containing 10.0 ml of water, which were promptly stoppered. After at least 24 hours, aliquots were assayed for C^{14} by a barium carbonate precipitation technique. The carbonate was plated on discs of Whatman no. 50 paper and the latter were pasted onto aluminum planchets. It was possible to keep the sample thickness uniform and below 0.4 mg/cm², and self absorption was negligible.

At the end of the incubation period the flasks were quickly chilled to 0° C. The radioactive solution then drawn off, and the slices washed for 15 minutes in several changes of 10^{-4} M calcium sulfate at 0° to remove radioactivity from the free space.

EXTRACTION: The chilled discs were dropped into 50 ml of boiling 80 % ethanol and were extracted for 30 minutes. Five more similar extraction periods followed. Extracts were combined and stored at −20° C until fractioned. This procedure extracted about 92 % of the total incorporated radioactivity from the tissue. Residual activity could not be extracted with boiling 20 % ethanol or boiling water.

Alcohol was removed by vacuum evaporation. Aqueous residues were diluted to about 25 ml and clarified by centrifugation if turbid. Fatty substances were removed by extraction with petroleum ether.

FRACTIONATION: Initial separation into basic, acidic, and neutral fractions was achieved by using two superimposed ion exchange resin columns. The columns (12 mm inside diameter) and a sample reservoir were made into a closed system, with ground glass joints. Design was such that solutions did not contaminate the joints, which were sealed with water only.

The upper column contained Amberlite IR–120(H) (a strongly acidic cation exchange resin) 16 to 50 mesh to a depth of 8 cm. The lower column was similarly packed with Amberlite IR–45(OH) (a weakly basic anion exchange resin) 16 to 50 mesh. Analytical grades of these resins, products of Rohm & Haas Co., Philadelphia, were used. Before use the resins were washed in turn with ethanol, concentrated ammonium hydroxide, 2 N sodium hydroxide, and 2 N hydrochloric acid. We found Amberlite IR–45 to have a significant sugar binding capacity. Labeled bound sugar was not removed by any eluting agent employed and could be recovered only very slowly by exchange with non-radioactive sugar. Consequently, isotopic recoveries in early fractionations ranged from 88 to 95 %. In later experiments losses due to sugar binding were greatly reduced by pretreating the resin with a solution containing 1 % each of glucose and fructose for 1 hour and then backwashing with water. After such treatment an average isotopic recovery of 98.75 % was attained in six successive fractionations.

Extracts, diluted to about 50 ml, were passed through the columns at the rate of about 1.5 ml per minute, followed by five bed volumes of water. The neutral fraction emerging from the second column was collected and evaporated to dryness under vacuum. All columns were eluted as soon as possible.

Amino acids and other bases were recovered from the IR–120 resin by elution with 50 ml of 4 N ammonium hydroxide passed through it at the rate of 1 to 2 ml per minute. The eluate was evaporated to dryness under vacuum.

Mixed acids were recovered from the IR–45 resin by elution with 400 ml of an ammonium carbonate-bicarbonate solution passed through at the rate of 2 to 3 ml per minute. This solution was prepared by dissolving 160 g of ammonium bicarbonate in water, adding concentrated ammonium hydroxide to pH 8.5 (about 55 ml), and diluting to 1 liter. The eluate was subjected to vacuum evaporation, whereupon the excess ammonium salts were volatilized. Just before the residue reached dryness a few milliliters of water were added to promote removal of remaining volatile salts. This was repeated if necessary. The final colorless residue consisted of ammonium salts of the mixed acids.

Paper strip electrophoresis was used to effect further separations of both basic and acidic fractions.

Hanging strip cells (28) made by Spinco Division, Beckman Instruments, Inc., were used along with Spinco papers.

The eluate from the IR–120 resin (amino acid fraction) was applied to six electrophoresis strips as a narrow band across the center of each. The electrolyte was 0.1 M ammonium formate adjusted to pH 6.0 with formic acid. These papers were subjected to 250 volts for 3 hours, which resulted in clean separation into discrete bands of acidic, basic, and neutral amino acids.

Paper electrophoresis was also used to separate the mixed acids into low pK (phosphorylated compounds) and high pK (carboxylic and other weak acids) groups. The electrolyte was 0.2 N formic acid (pH 2.2). Each sample (from 2.2 g of tissue) was distributed among eight paper strips as a narrow band 5.5 cm from the negative ends. The eight strip unit was subjected to a constant current of 12 milliamperes for about six hours. The two major bands were separated by 3 to 4 inches. Conversion to the free acid form occurred simultaneously.

Radioactive compounds on the papers were located by recording strip scanning devices. The corresponding active areas from the several papers of each sample were cut out and eluted groupwise between glass strips. Overnight elution with water by this method usually yielded 95 to 100 % recovery of labeled compounds.

The high pK acids were further fractioned by descending paper chromatography. Whatman 3 MM paper previously washed with a mixture of ethyl acetate, acetic acid, and water (10:5:2 by volume) was used. The first solvent was the organic phase of *n*-butanol, formic acid (88 %), and water mixed in equal parts by volume and aged for 24 hours. Resolution into seven discrete bands, as revealed by spraying with brom phenol blue, was attained in about seven hours. After strip scanning, the bands were eluted separately, and rechromatographed in other solvents or subjected to electrophoresis as necessary to provide separation and tentative identification. Citric and isocitric acids were separated by the acid amide technique of Ranson (18).

The sugar fractions were banded on 10 cm widths of Whatman no. 3 MM paper previously washed with the solvent. A mixture of ethyl acetate, acetic acid, and water (10:5:2 by volume) was used to develop the papers (descending). Radioactive bands were rechromatographed as necessary. Para-anisidine (16) was used as an indicator. The entire area beyond fructose was eluted and the eluate rechromatographed as a narrow band. Thus the small amount of activity in pentoses and other high R_f sugars was easily revealed. The individual sugars in this group were not identified.

The low pK acid fraction, mostly sugar phosphate esters, was not examined in detail.

COUNTING TECHNIQUES: Fractionation procedures were monitored by radioactivity determinations after each step. Samples were taken by evaporating solutions to dryness under vacuum, redissolving in a known volume, and removing duplicate aliquots (1 or 2 % of the total) for counting. Self absorption was not a problem when small samples were used. Samples were plated on glass planchets and counted under a thin window gas-flow detector (D–47, Nuclear-Chicago Corp.). Counting efficiency was maintained at 27 % by frequent use of standards and adjustment of the distance between detector and sample as necessary.

Residual radioactivity in extracted tissue was assayed by oxidizing aliquots by a wet combustion technique (29). The carbon dioxide was trapped in 1 M hydroxide of Hyamine 10-X (*p*-[diisobutyl-cresoxyethoxyethyl]-dimethylbenzylammonium hydroxide) in methanol (prepared by Packard Instruments Co., Inc., La Grange, Ill.). Aliquots of the Hyamine carbonate solution were counted with a Packard Tri-Carb Liquid Scintillation Spectrometer using a scintillator and solvent system based upon that recommended by Hours and Kaufman (8) for tritium. Results obtained at a counting efficiency of 32 % were corrected to 27 % after calibration with standards.

EXPERIMENTAL RESULTS

RESPIRATORY RISE: Potato tuber slices incubated at room temperature for 30 hours exhibit an approximately fourfold increase in respiratory rate. However, similar to the metabolic changes which occur in aging chicory root slices (11), the oxygen uptake of fresh slices is inhibited only about 15 % by 0.05 M

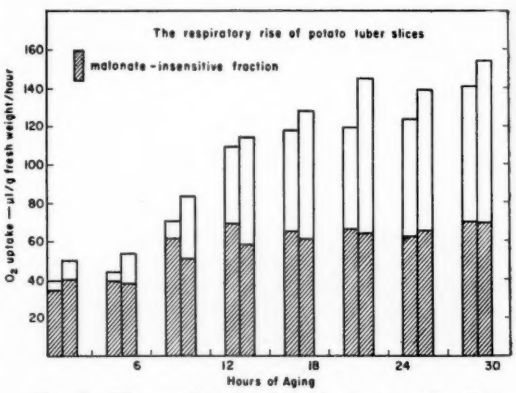


FIG. 1. The respiratory rise of potato tuber slices. After incubation in 10^{-4} M $CaSO_4$ solution for the indicated period the O_2 uptake of samples was measured by conventional manometric techniques at 28° C. Malonate treatment consisted of suspending in test solution containing 5×10^{-2} M potassium malonate (pH 5.0) and 10^{-4} M $CaSO_4$. Controls were suspended in a similar solution containing 5×10^{-2} M potassium chloride but also 5×10^{-4} M potassium malonate at pH 5.0. Slices were put into test solutions 15 minutes before zero time. Each pair of bars represents a separate Warburg run with each treatment triplicated.

malonate at pH 5.0, whereas that of aged slices is inhibited by about 50 %. Thus during the incubation period the malonate resistant fraction of the respiration doubles whereas the malonate sensitive fraction increases 10-to 15-fold (fig 1).

Absorption & Utilization of Radioglucose as Function of Aging: Fresh potato slices, in contrast to aged, have little ability to absorb phosphate (6, 14) and salts in general (10, 13). We found an even greater disparity in the ability of fresh and aged slices to take up and oxidize glucose.

Fresh slices release $C^{14}O_2$ from randomly labeled glucose at a slow rate not initially reduced by treatment with malonate. Slices aged for 36 hours before being presented with labeled glucose release $C^{14}O_2$ at a rate which, during the first 30 minutes, is about 3,000 times greater than that of fresh tissue. In aged tissue, malonate greatly inhibits the evolution of $C^{14}O_2$ from labeled glucose so that it is only 150 to 180 times more rapid than in similar fresh tissue (table I). Hence malonate has no initial inhibitory effect upon $C^{14}O_2$ output in fresh slices but reduces output by aged slices 12-to 15-fold. This occurs even though total respiratory O_2 uptake is reduced by only about 50 % (fig 1). The malonate effect upon $C^{14}O_2$ output in aged slices is the compound result of inhibition of uptake and utilization. In the experiment cited (table I), control and malonate treated slices after 3 hours of incubation in radioglucose solution had taken up 10,800,000 and 2,840,000 cpm, respectively, from 12,000,000 cpm available to each. These results clearly show malonate inhibition of glucose uptake, but this alone is insufficient to account for the observed effect. The remainder is due to inhibition of utilization. Collectively the data suggest that qualitative, rather than merely quantitative, metabolic changes characterize the physiological aging process.

Table I

Oxidation of Exogenous Radioglucose* by Freshly Cut & 36-hr Aged Potato Slices**

Min after zero time	cpm*** in Respiratory CO_2		Ratio
	Fresh	Aged	Aged / Fresh
Controls†			
0–10	74	269,000	3640
10–20	121	398,000	3280
20–30	169	455,000	2690
30–40	214	446,000	2080
Malonate treated†			
0–10	94	17,500	186
10–20	174	30,800	177
20–30	199	36,100	181
30–40	265	38,500	145

* Supplied as 20 μC (approx 12,000,000 cpm) of randomly labeled glucose-C^{14}, specific activity 45.9 μC/μM, in 7.5 ml of test solution ambient to 40-disk tissue samples (2.2 g fresh wt). Initial glucose concentration was 5.8×10^{-5} M.

** Fresh slices pre-incubated in non-radioactive test solution for 1 hr at 0° C, aged slices similarly pre-incubated during the 36th hour at room temperature.

*** Counted with a thin window, gas-flow detector at approximately 27 % efficiency.

† Malonate treated disks suspended in test solution containing 5×10^{-2} M potassium malonate (pH 5.0) and 10^{-4} M $CaSO_4$. Controls suspended in similar solution having 5×10^{-2} M potassium chloride but also containinging 5×10^{-4} M potassium malonate to stabilize pH at 5.0.

Labeling Patterns in Fresh Tissues: By using 10 to 20 μC of high specific activity glucose in 7.5 ml of solution suspending 2.2 g of fresh slices, it was possible to incorporate useful amounts of radioactivity into metabolic intermediates during 1 to 3 hour incubation periods in spite of slow uptake and utilization (tables II, III, & IV). Our results suggest that, if pursued, this approach could yield a great amount of information about the malonate resistant respiration of fresh slices. Such further work will be facilitated by the fact that tissues incubated in the presence of malonate maintain their fresh characteristics for long periods because malonate, like low temperature, prevents the development of the respiratory increment normally induced by aging.

In accord with earlier reports that a rapid sucrose synthesis begins when potato tubers are cut (7, 17), we found that more than half the total extracted activity from fresh slices, following the absorption of uniformly labeled glucose, was in sucrose (table II). Hydrolysis of the sucrose yielded monosaccharides of equal activity. The free fructose was, however, much less active than free glucose (table II) although they were present in similar amounts. In short term experiments with fresh tissue, sucrose behaved as an end product rather than as part of the substrate pool.

Although malonate had only a small inhibitory effect upon sucrose synthesis (table II), it decreased total activity in the acidic and basic fractions and had the indirect effect of increasing the percentage of the total activity appearing in sugars (table IV). Sucrose synthesis in the presence of malonate indicates that the malonate resistant respiration provides energy for some synthetic activity although its ability to support active uptake of glucose and of salts is very limited.

A pronounced accumulation of succinic acid in slices which had been incubated with labeled glucose in the presence of malonate for 3 hours was evident from inspection of chromatograms and from counting data (table II). This indicates that malonate exerts its classical effect upon succinic dehydrogenase in tissue aged for less than 3 hours, although oxygen uptake is little inhibited. It is important to note, however, that in similar experiments of 1 hour duration, malonate caused no obvious increase in amount of succinic acid, and only about a 50 % increase of activity therein. Thus a disproportionate amount of the succinate accumulation occurs during the 2nd and 3rd hours. Hence the metabolic changes induced by aging appear to begin during the first 2 hours. This

idea is supported by the results of our experiments with specifically labeled glucose and by Loughman's work on phosphate uptake by potato slices (14). Further experiments on label distribution after only 1 hour of incubation seem desirable.

EXPERIMENTS WITH SPECIFICALLY LABELED GLUCOSE: Identical 40-slice samples of fresh tissue were incubated for 3 hours in the presence of glucose-1-C^{14}, glucose-6-C^{14}, and randomly labeled glucose (glucose-R-C^{14}), respectively. All the sugars were adjusted to a specific activity of 4.6 $\mu C/\mu M$. A total of 10 μC of radioactivity (approx 6,000,000 cpm) was used in each experimental flask which contained 7.5 ml of test solution having a total initial glucose concentration of 2.9×10^{-4} M. Histograms plotted on the basis of radioactivity in respiratory CO_2 collected during 18 successive 10 minute periods show that the respiratory mechanisms of fresh slices discriminate between the first (C_1) and sixth (C_6) carbon atoms of the glucose molecule both in the controls and malonate treated samples (figs 2 & 3). The ratio of the radioactivity released from glucose-1-C^{14} to that released from glucose-6-C^{14} (C_1/C_6 ratio) was initially high for both control and malonate treated samples and dropped to about three in both after 1 hour. Thereafter, however, the malonate treated slices exhibited an increasing ratio (fig 3). The 3 hour over-all C_1/C_6 ratios of 2.67 and 3.75 for control and malonate treated samples, respectively, reflect the inhibition of C_6 release by malonate, but do not reveal that it is confined mostly to the 3rd hour.

Aged slices also were incubated with glucose-1-C^{14}

TABLE II

EFFECT OF MALONATE UPON DISTRIBUTION OF RADIOACTIVITY FROM RANDOMLY LABELED GLUCOSE* IN FRESH POTATO SLICES**

COMPONENT***	cpm†		MALONATE TREATED AS % OF CONTROL
	CONTROL	MALONATE TREATED	
Respiratory CO_2	22,600	17,300	77
Total ethanol extract	705,000	567,000	80
Lipids	111	367	330
Basic Fraction	96,500	73,700	76
Acidic amino acids	56,100	16,700	29
Neutral amino acids	32,100	56,200	175
Basic amino acids	950	750	79
Acidic fraction	99,400	54,400	55
Phosphorylated compounds	28,000	10,000	36
Weak acids	67,300	41,500	62
Low R_f unknowns	43,300	24,950	58
Citric	4,350	1,280	29
Isocitric	235	757	322
Unknown	102	505	495
Malic	8,900	1,740	20
Glycolic	2,750	1,830	67
Malonic and unknown	205	330	161
Succinic	925	6,300	682
Fumaric	190	125	66
Neutral fraction	399,000	361,000	91
Oligosaccharides	8,050	6,000	75
Sucrose	344,000	306,000	89
Glucose	31,900	26,800	84
Fructose	5,950	5,830	98
High R_f sugars	< 400	< 400	...

* Supplied as described in same footnote table I.

** Slices were preincubated for 1 hour in 10^{-4} M $CaSO_4$ at 0° C then brought to room temperature and incubated for 3 hours in the test solution containing labeled glucose.

*** The respiratory CO_2 was trapped on alkali papers then converted to $BaCO_3$ and counted. Various components were isolated from 80 % ethanol extracts by means of ion exchange, paper electrophoresis, and paper chromatography.

† Duplicate samples on glass planchets were counted with a thin window, gas flow detector. Recoveries were incomplete because only the more active areas of papers were eluted. Counting efficiency was approximately 27 %.

TABLE III

UPTAKE & DISTRIBUTION OF RADIOACTIVITY FROM LABELED GLUCOSE* BY FRESH POTATO SLICES** INCUBATED 3 HOURS WITH & WITHOUT MALONATE

TREATMENT	cpm IN THOUSANDS***			
	RESPIRED CO_2	TOTAL EXTRACT	RESIDUAL IN TISSUE	TOTAL UPTAKE
Control†				
Glucose-1-C^{14}	35.1	462	47.1	544
Glucose-6-C^{14}	13.1	534	33.0	590
Glucose-R-C^{14}	16.7	497	47.0	561
Malonate†				
Glucose-1-C^{14}	30.2	434	28.6	493
Glucose-6-C^{14}	8.1	497	50.1	555
Glucose-R-C^{14}	18.5	405	33.0	457

* Supplied as 10 μC (approx 6,000,000 cpm) of radioactive glucose of the specified labeling pattern in 7.5 ml of test solution ambient to 40 slice tissue samples (2.2 g fresh wt). Initial glucose concentration was 2.9×10^{-4} M.
** Slices were preincubated in non-radioactive test solutions for 1 hour at 0° C, then quickly brought to room temperature and presented with radioactive solutions.
*** See same footnote table I.
† See same footnote table I.

and glucose-6-C^{14}. Because aged slices evolve $C^{14}O_2$ from exogenous glucose several thousand times more rapidly than fresh slices (table I), only 0.4 μC (about 240,000 cpm) instead of 10 μC was used per sample. Specific activities were adjusted to 2.19 μC/μM and the initial total exogenous sugar concentration was 2.44×10^{-5} M in each flask. These experiments were run for only 1 hour.

As in fresh tissue, $C^{14}O_2$ release by aged slices was much more rapid from glucose-1-C^{14} than from glucose-6-C^{14}. That the release of label from aged malonate treated slices was much less than from aged controls (fig 4) was the combined result of malonate inhibition of respiration and of glucose uptake. However, because no discrimination is to be expected during uptake, this inhibition should have no effect upon the C_1/C_6 ratio itself. This ratio was about 4 in the control and about 15 in the malonate treated slices (fig 5).

DISCUSSION

Participation of the PPP in respiratory metabolism may be demonstrated by a technique, introduced by Bloom and Stetten (4), employing glucose-C^{14} preparations specifically labeled in the first (C_1) and sixth (C_6) carbon atoms, respectively. This is possible because, during short times at least, the oxidation and decarboxylation of glucose molecules to pentose phosphate leads to evolution of CO_2 arising specifically from C_1, whereas in the case of molecules degraded to trioses by the Embden-Meyerhof-Parnas pathway (EMPP), and subsequently oxidized, C_1 and C_6 are equivalent and appear in respiratory CO_2 at equal rates. Of course, if the added labeled sugar were completely oxidized during the experimental period, the over-all ratio would be unity regardless of the pathway. Thus it is the ratio and its changes during the early stages of feeding experiments which are significant.

A factor which may lead to elevated C_1/C_6 ratios when both PPP and EMPP-TCAC are present is the incorporation of glycolytic products into compounds which are not part of the metabolic pool. In the presence of PPP such sequestering can raise the C_1/C_6 ratio by retarding release of label from that portion of hexose which has entered the EMPP. In our opinion this effect is of importance only when an attempt is being made to measure relative glucose degradation by the several pathways. We are interested in their relative contributions to respiratory gas exchange. If during a short time period a large fraction of the glycolytic product is being sequestered rather than oxidized via TCAC, the EMPP-TCAC system is in fact making a reduced contribution to respiration (as distinct from glucose degradation) just as indicated by the raised C_1/C_6 ratio for that time period.

We recognize that the C_1/C_6 ratio cannot be used to calculate the fraction of the total respiration proceeding by a particular pathway, and also that a single measurement of the ratio without regard to its rate of change cannot have much validity. Yet the ratio, if it exceeds unity, speaks for the existence of a pathway other than EMPP-TCAC and gives an estimate of the relative contribution of EMPP-TCAC and PPP to the release of CO_2 from C_1 of glucose (when expressed as $\frac{C_1-C_6}{C_1}$). For these reasons we have made ratio determinations at frequent intervals and have based our interpretations mostly upon relative changes with time rather than upon absolute values.

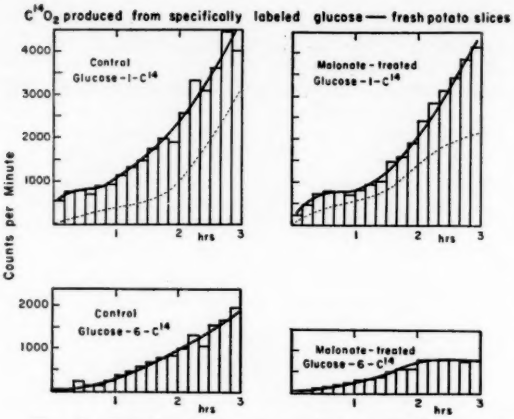


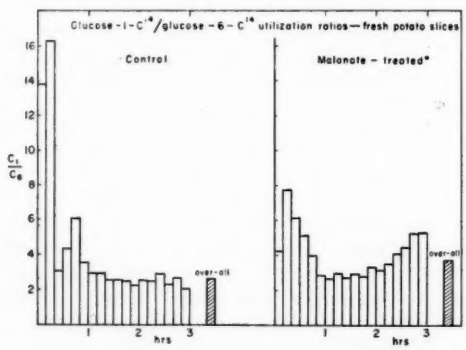
FIG. 2. Relative rates of respiratory $C^{14}O_2$ output from fresh potato slices incubated with specifically and randomly labeled glucose. The dashed curves represent results obtained with the latter. Experimental conditions were as described in the footnotes for table III.

We advance the following tentative interpretations as a basis for further experiments:

In fresh tissue, during the first and part of the 2nd hour, CO_2 release from glucose is mediated largely by the PPP. Although the EMPP may also be functional (1), release of C_6 from glycolytic products is slow. The fact that evolution of $C^{14}O_2$ from glucose-R-C^{14} rises rapidly during the first hour (6.3-fold increase) whereas the increase in label release from glucose-1-C^{14} is less rapid (1.7-fold increase) suggests that glycolytic products contribute increasingly with time. During the first three 10 minute intervals the ratio of label released from glucose-R-C^{14} to that from glucose-1-C^{14} was 1/5.3 (fig 2)—close to the theoretical of 1/6 for oxidation by the PPP exclusively—and in accordance with the suggestion that the PPP predominates initially. The drop in C_1/C_6 ratio from a high of 16.3 to 3.6 during the first hour (fig 3) appears to be the consequence of the onset of oxidation of glycolytic products either via the TCAC or via some other malonate resistant pathway.

In view of the locus of malonate inhibition, malonate would be expected to inhibit the release of radioactivity from glucose-R-C^{14} oxidized via the TCAC more readily than release via the PPP. Because C_1 release is not inhibited appreciably by malonate during 3 hours of incubation, but label release from glucose-R-C^{14} is inhibited considerably after 2 hours (fig 2), it appears that PPP and TCAC are somewhat competitive and that PPP becomes increasingly active as TCAC is inhibited. This may explain why the label distribution after 3 hours of incubation (table II) indicates considerable TCAC activity even though malonate inhibition of O_2 uptake is small. During aging malonate resistant respiration doubles while the total respiration, increasingly malonate sensitive, rises four-fold (fig 1). In aged tissue PPP apparently can no longer compensate for the large TCAC mediated fraction after the latter has been inhibited by malonate, inhibition of O_2 uptake then becomes evident, and the C_1/C_6 ratio rises. This rise in ratio begins to be evident during the 2nd hour (fig 3).

In the fresh controls, the rise in label evolution from glucose-R-C^{14} was as steep as for glucose-1-C^{14} (fig 2). Because equal quantities of label were administered and uptake was similar (table III), then, barring rapid randomization effects for which we have no evidence, the theoretical yield of $C^{14}O_2$ from glucose-R-C^{14} would be about one-sixth that from glucose-1-C^{14} if PPP predominated. Although the ratio was approximately one-sixth during the 1st hour, it rose until it was roughly three-fourths at the end of 3 hours. Again the implication is an increasing contribution by the TCAC.



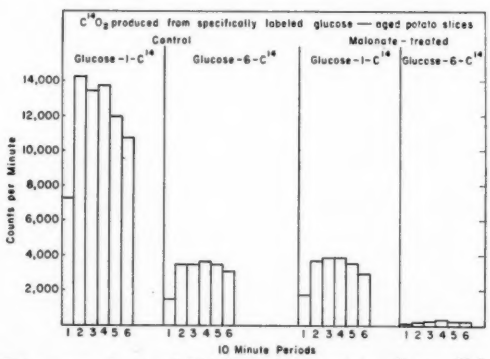


FIG. 3 (*left*). Ratio of $C^{14}O_2$ released from glucose-1-C^{14} versus glucose-6-C^{14} from fresh potato slices. Calculated from the data represented by figure 2.

FIG. 4 (*right*). Relative rates of $C^{14}O_2$ output from potato slices aged for 30 hours in 10^{-4} M $CaSO_4$ and then supplied with glucose specifically labeled on C_1 or C_6. The glucose was dissolved in test solutions of the formulation given in the legend to figure 1.

TABLE IV

DISTRIBUTION OF RADIOACTIVITY FROM LABELED GLUCOSE IN EXTRACTS OF FRESH POTATO SLICES INCUBATED 3 HOURS WITH & WITHOUT MALONATE*

TREATMENT	% INITIAL TOTAL EXTRACT			
	BASES	ACIDS	SUGARS	TOTAL RECOVERED
Controls				
Glucose-1-C^{14}	14.3	14.6	70.1	99.0
Glucose-6-C^{14}	17.0	14.9	65.0	96.9
Glucose-R-C^{14}	12.6	13.2	72.0	97.8
Malonate				
Glucose-1-C^{14}	12.8	10.1	75.6	98.5
Glucose-6-C^{14}	16.5	12.3	72.2	101.0
Glucose-R-C^{14}	12.0	9.6	77.6	99.2

* All experimental conditions as described in footnotes to table III.

In the case of glucose-6-C^{14} the inhibitory effect of malonate upon the rise in release of label after 2 hours (fig 2) is striking. Malonate simultaneously depresses release of label from glucose-R-C^{14}, but the latter effect is less evident because randomly labeled hexose releases label from C_1 also.

In fresh controls there was a sharp drop in the C_1/C_6 ratio during the first 2 hours (fig 3). In view of the response to malonate it is likely that this is largely the result of onset of TCAC activity. In the presence of malonate the development of TCAC activity is inhibited and the drop in C_1/C_6 ratio is soon reversed.

Studies of isotope distribution after fresh slices had been incubated with labeled glucose for 3 hours revealed that considerable C^{14} had been sequestered into non-extractable cell material (table IV) or in extractable compounds probably not on direct pathways to CO_2 evolution (table II). Such sequestering is not disadvantageous because in these short term experiments we are not concerned with recovery of large fractions of the added isotope as $C^{14}O_2$. Sequestering of glycolytic products, for reasons mentioned above, is also of minor importance. In any case the ratio $(C_1-C_6)/C_1$ is an expression of the relative contributions of PPP and EMPP-TCAC to the release of label from glucose-1-C^{14}, and thus gives a partial measure of the relative activity of the two pathways in respiration. When the data from figure 2 are expressed on this basis, the percentage contribution of PPP to C_1 release in the controls falls from an initial above 90 to about 70 in 1 hour and then levels off at 60 to 65. In the presence of malonate, the relative contribution of PPP is also initially high and falls to 60 to 65 during the 2nd hour, but then rises to 80 again during the 3rd hour. This is consistent with the idea of onset of TCAC during the 2nd hour. A complicating factor is the presence of a malonate resistant C_6 releasing mechanism throughout the experimental period (fig 2).

There is no evidence of any significant malonate inhibition of label release from glucose-6-C^{14} until the 2nd hour, and the C_1/C_6 ratio then rises (fig 3). The lack of malonate inhibition of label release from glucose-R-C^{14} or glucose-6-C^{14}, lack of succinate accumulation, and the high initial C_1/C_6 ratio all indicate that TCAC activity is negligible during the 1st hour. The observed C_6 release suggests a mechanism other than the TCAC. It could represent release via PPP from hexose made from triose fragments (2), but the possibility of malonate resistant pathways other than the PPP being operative in C_6 release cannot be dismissed. Laties (12) has presented evidence for the existence of two types of malonate resistant respiration in aging chicory root slices. This may be true for potato slices also.

In aged tissue the inhibitory effect of malonate upon C_1 release (fig 4) at first glance suggests that about two-thirds of the total is due to a malonate sensitive system, presumably TCAC. Thus, paradoxically, it seems that C_1 release via the TCAC is about twice as great as total C_6 release in the aged control. The apparent paradox results from inhibition by malonate of glucose uptake per se in aged slices. Such inhibition of uptake, of course, applies equally to C_1 and C_6 and does not alter the C_1/C_6 ratios.

The C_1/C_6 ratio of aged control slices (fig 5) after 1 hour in the presence of labeled glucose is similar to that of fresh tissue after exposure for an equal period (fig 3). But it is not valid to argue that the contribution of the several pathways to respiration is, therefore, the same. Whereas in fresh tissue C_6 release during the 1st hour is not noticeably inhibited by malonate, in aged tissue malonate reduces C_6 release much more than C_1 release and the C_1/C_6 ratio becomes very high (fig 5). These results, together with those of manometric experiments (fig 1) revealing a 10-to 15-fold increase in malonate sensitive respiration during aging, speak for the existence of

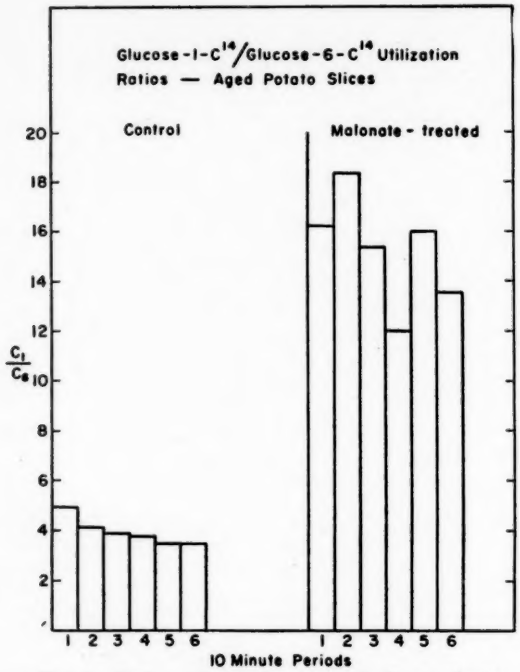


FIG. 5. Ratios of $C^{14}O_2$ released from glucose-1-C^{14} versus glucose-6-C^{14} by 30 hour aged potato slices. Calculated from the data represented by figure 4.

a malonate sensitive, non-discriminating pathway in aged tissue which is initially inoperative in fresh tissue. Thus the respiration of aged slices appears to consist of two major fractions, one, probably EMPP-TCAC, can be inhibited by malonate, the other, probably PPP or related system, is little affected by malonate and accounts for the bulk of the remaining respiratory capacity of aged tissue in the presence of malonate.

The enzymes of the TCAC are not lacking in fresh tissue. Furthermore, it is known that mitochondria from fresh and aged tissue oxidize TCAC acids at similar rates, and also that aging is not accompanied by any measurable increase in cytochrome c oxidase activity (6). Lack of malonate inhibition in fresh and of cyanide inhibition in aged tissue cannot, therefore, be adequately explained by an hypothesis requiring greatly over-dimensioned TCAC enzymes in fresh tissue and a gross excess of cytochrome c oxidase in aged. The fact that slices from newly harvested tubers contain glycolic acid, but lack the acids of the TCAC, whereas the latter appear during aging (Laties, pers communic), is further evidence that the TCAC is inoperative in fresh tissue. The reason for the initial inactivity of the TCAC, even when the acids of the cycle are present, is not obvious. Perhaps ancillary enzymes or cofactors are missing or an inhibitor of an essential step is present.

In contrast to ap Rees and Beevers (1), we believe that the major change which occurs during aging of potato slices is the activation of a malonate sensitive respiratory pathway—presumably TCAC. This development begins early in the aging period so that after only 2 hours the tissue can no longer be considered as strictly fresh. In truly fresh tissue TCAC activity is minimal, and respiratory CO_2 release is mediated primarily via PPP. Both PPP and TCAC activity increase with time, as does sensitivity to malonate. It is especially significant that malonate immediately raises the C_1/C_6 ratio in aged tissue, while in fresh tissue it has little effect until the 3rd hour. It is interesting that when slices are incubated in the presence of malonate, physiological aging does not accompany chronological aging and fresh characteristics can be maintained for long periods. If the major change induced by aging were increased PPP activity unaccompanied by the development of TCAC activity, the observed malonate effects would not be expected.

On the basis of experience gained from this work we believe that this approach can yield valuable information about slowly respiring tissues. Our results indicate that changes in the relative activities of the several respiratory pathways can occur rapidly and that frequent measurements during the early hours of aging are necessary. Total uptake information as a function of time, data on specific activity changes in various metabolic pools, and detailed studies of the distribution of label among metabolic intermediates would be valuable adjuncts; however, we have not been able to continue the work.

SUMMARY

The respiration of freshly cut 1 mm thick potato tuber slices is only slightly inhibited by 0.05 M malonate at pH 5.0. However, during incubation of the slices at room temperature sensitivity to malonate increases. During 30 hours of such physiological aging, total respiration increases four- to fivefold, the malonate resistant fraction doubles, and the malonate sensitive fraction increases 10- to 15-fold over its activity during the 1st hour.

Fresh slices have a very limited ability to absorb and oxidize exogenous glucose, as measured by evolution of $C^{14}O_2$ after feeding labeled glucose. This ability increases more than 3000-fold during 36 hours of aging; however, whereas $C^{14}O_2$ evolution by fresh tissue is not inhibited by malonate, that of aged tissue is reduced by a factor as high as 15.

In spite of limited uptake by fresh slices, techniques were developed which, after use of high specific activity C^{14} labeled glucose as a tracer, permit recovery of labeled metabolic intermediates after 1 to 3 hours of incubation. A method of fractioning extracts is described which by means of ion exchange, paper electrophoresis, and paper chromatography allows isolation of amino acids, carboxylic acids, sugar, and phosphorylated compounds from the same sample.

Glucose-1-C^{14}, glucose-6-C^{14}, and randomly labeled glucose, were fed to tissue slices in the presence and absence of malonate. Respiratory CO_2 was collected and C_1/C_6 ratios were calculated for each 10 minute interval for up to 3 hours. In such experiments fresh slices show initially high but declining C_1/C_6 ratios. Malonate has little effect at first, but reverses the decline during the 2nd hour. By contrast, in aged slices malonate causes an immediate and pronounced increase in the ratio from a control value of about 4 to about 16.

The following tentative interpretations of the data are advanced as a basis for further experimental work: The respiration of fresh slices is largely mediated by the pentose phosphate pathway. Within 2 hours products of glycolysis begin to be oxidized via the malonate sensitive tricarboxylic acid cycle. In aged tissue also the pentose phosphate pathway or similar system is operative, but the tricarboxylic acid cycle mediates a large fraction of the total respiration. The appearance of tricarboxylic acid cycle activity within 3 hours and its continued development to become a major component of respiration is probably the major fundamental change occurring during physiological aging. The rapidity of change during the early hours of incubation must be considered in designing experiments.

Acknowledgments

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ISOLATION AND CHARACTERIZATION OF MALFORMIN[1,2,3]

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Papers (5, 6) from this laboratory have reported the induction, by culture filtrates of the fungus, *Aspergillus niger*, of curvatures and malformations of bean plants and curvatures of corn roots. The name, malformin, was proposed for the active compound (7). We now present a complete isolation procedure for malformin and describe, in part, the character of the molecule. Isolation of malformin has made it possible to conduct quantitative experiments on its activity (see following report).

MATERIALS & METHODS

Aspergillus niger van Tiegh. strain 56-39 was used in these and the original studies. The culture medium, corn steep-glucose, and shake-flask methods were described earlier (5). Culturing was done at 25 to 26° C.

Cultures of 56-39 were obtained from soil stocks by inoculating agar slants of potato-dextrose medium. After 7 to 10 days, one ml spore suspensions were used to inoculate 100 ml aliquots of corn steep-glucose medium in 500 ml erlenmeyer flasks. After 24 hours on a reciprocating shaker, the cultures were transferred to additional 100 ml aliquots of the same medium in 1 liter erlenmeyer flasks and again incubated for 24 hours on the shaker. Each of these cultures was transferred to New Brunswick Model FS-314 fermentation jars containing 10 liters of corn steep-dextrose medium. The jars were incubated for 4 to 5 days on a New Brunswick fermentor drive assembly (air flow = 6 liters/min/jar; agitation speed = 420 rpm). Antifoam (Dow Polyglycol P-2000) was added as needed. At the end of the fermentation period the mycelium was removed by filtration and discarded.

To follow the isolation of malformin from the filtrates, one of two methods of assay was employed, the bean test (5) or the corn root test (6). The fractions were usually dried and then dissolved in water (containing 4 drops of Tween 80/100 ml for the bean test). In some cases a few drops of acetone were added to the dried residue to assist in dissolving the material.

Additional methods are given later.

RESULTS

I. PURIFICATION OF MALFORMIN: A schematic outline (fig 1) presents the various stages of isolation. After adding activated charcoal to the culture filtrate (step 1) the charcoal-filtrate mixture was stirred for 1 hour. The charcoal settled overnight and was separated from the cleared filtrate by decantating. Additional water was removed by filtering through Whatman no. 1 filter paper. After drying, the charcoal was made into a slurry with technical grade 99 % acetone and poured into a 7 × 90 cm column (step 2). The column was eluted with more acetone. About 1.5 liters of acetone were used for each 100 g of air dried charcoal. When the acetone was removed by distillation (step 3) an aqueous residue remained, its volume depended on the water content of the acetone and the charcoal. This residue was made alkaline (pH 8-9) by adding saturated $NaHCO_3$ solution, or by adding the bicarbonate directly to the aqueous residue. Malformin was removed from the aqueous phase by five extractions with ethyl ether. The ether was then extracted twice with 30 % (v/v) H_2SO_4 to remove basic substances; malformin remained in the neutral ether fraction (step 4). The 30 % H_2SO_4 solution was again extracted with ether twice, and the ether was combined with the neutral ether fraction containing malformin (step 5). Anhydrous Na_2SO_4 was added in excess to the neutral ether fraction for drying (step 6). After filtering and evaporating the ether a yellow syrup remained. By washing this syrup with small quantities of absolute acetone most of the malformin remained as a crude white powder, whereas the yellow substance was dissolved in the acetone. Although malformin is slightly soluble in acetone the use of small quantities minimized the loss. A second crop of crude white powder was usually recovered from the acetone by allowing it to evaporate for 3 to 4 days. Most of the crude white powder, about 600 mg from 30 liters of culture filtrate, could be dissolved by refluxing five times for 30 minutes with 50 ml aliquots of ethyl acetate (reagent grade, step 7). Insoluble material was removed by filtration. The ethyl acetate solution was condensed to approximately 100 ml and added to a 2.2 × 40 cm column of activated alumina (Merck, reagent grade) previously packed with ethyl acetate. The column was eluted with approximately 300 ml of ethyl acetate at a rate of 20 to 30 drops/min. Evaporation of this fraction yielded a small quantity of syrup which had no activity on bean plants. The eluting solvent was then changed to ethyl acetate containing 2 % (v/v) ethyl alcohol. After 150 ml

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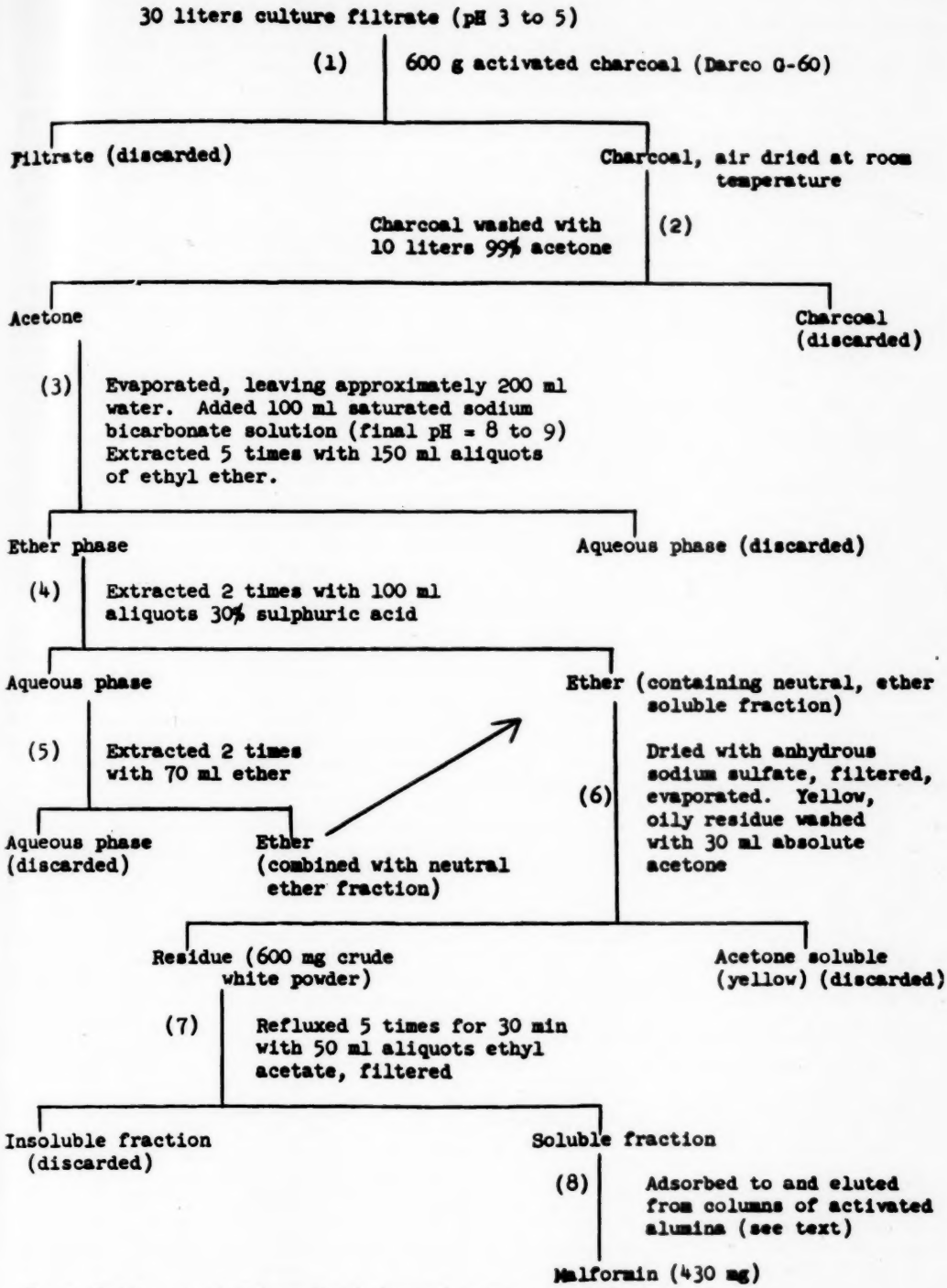

30 liters culture filtrate (pH 3 to 5)
(1)
600 g activated charcoal (Darco G-60)
Filtrate (discarded)
Charcoal, air dried at room temperature
Charcoal washed with 10 liters 99% acetone
(2)
Acetone
Charcoal (discarded)
(3)
Evaporated, leaving approximately 200 ml water. Added 100 ml saturated sodium bicarbonate solution (final pH = 8 to 9) Extracted 5 times with 150 ml aliquots of ethyl ether.
Ether phase
Aqueous phase (discarded)
(4)
Extracted 2 times with 100 ml aliquots 30% sulphuric acid
Aqueous phase
Ether (containing neutral, ether soluble fraction)
(5)
Extracted 2 times with 70 ml ether
(6)
Dried with anhydrous sodium sulfate, filtered, evaporated. Yellow, oily residue washed with 30 ml absolute acetone
Aqueous phase (discarded)
Ether (combined with neutral ether fraction)
Residue (600 mg crude white powder)
Acetone soluble (yellow) (discarded)
(7)
Refluxed 5 times for 30 min with 50 ml aliquots ethyl acetate, filtered
Insoluble fraction (discarded)
Soluble fraction
(8)
Adsorbed to and eluted from columns of activated alumina (see text)
Malformin (430 mg)

FIG. 1. Isolation procedure for malformin from culture filtrate of *A. niger* 56–39.

had been collected, the solvent was evaporated and 430 mg of a pure white powder remained (from 30 liters of culture filtrate). Recent evidence indicated that using 2% alcohol in the eluting solvent may not be necessary. The white powder was active on bean seedlings or corn roots when as little as 1×10^{-4} μg/plant were used. Adsorption and elution from alumina columns was repeated for further purification. Malformin did not melt but did decompose at temperatures over 300° C.

The movement of malformin in the solvents indicated that the molecule had neither acidic nor basic groups, and no amphoteric properties; thus, the compound was neutral. These results agree with earlier studies indicating that malformin could not be adsorbed by anion or cation exchange resins (5).

II. Molecular Formula: From the analysis of malformin (table I) for carbon, hydrogen, nitrogen, and sulfur the experimental formulae $(C_{23}H_{39}N_5O_5S_2)_n$ or $(C_{23}H_{41}N_5O_5S_2)_n$ were calculated. They differ only in the number of hydrogen atoms. It was impossible to differentiate the two by the results of elemental analysis.

The molecular weight of malformin was determined by the Barger method (2). A solution of malformin was equilibrated with various concentrations of standard solutions of recrystallized acetanilide in capillary tubes. In two determinations the molecular weight was 530 ± 45 and 495 ± 45. These values agree with the formulae of $C_{23}H_{39}N_5O_5S_2$ or $C_{23}H_{41}N_5O_5S_2$ (table I).

Table I

Elemental Analysis of Malformin

	% Carbon	% Hydrogen	% Nitrogen	% Sulfur	Molecular weight
Found	52.19	7.53	13.35	11.90	512*
$C_{23}H_{39}N_5O_5S_2$	52.11	7.36	13.22	12.10	530
$C_{23}H_{41}N_5O_5S_2$	51.88	7.71	13.16	12.06	532

* Average of two experiments.

III. Physical & Chemical Properties of Malformin:

A. Infrared Spectrum: A Perkin-Elmer Model 21 Spectrophotometer was used for the infrared studies. The infrared spectrum (fig 2) of malformin showed strong adsorption peaks at 3.01, 6.06, and 6.54 μ. These adsorption bands are typical of peptides.

B. Ultraviolet Spectrum: The ultraviolet spectrum was taken on a Beckman Model DU spectrophotometer. Solutions of malformin were prepared with methyl cellosolve and diluted with water. In the region from 220 to 330 mμ no adsorption peaks were observed. End adsorption was observed at the shorter wave lengths (at 220 mμ, $\epsilon = 5400$). The lack of ultraviolet adsorption bands excluded the possibility of any conjugate system in the molecule.

C. Optical Rotation: A 1.1% solution of malformin in methyl cellosolve was used for optical rotation determinations. Specific rotation was calculated from the readings to be $[\alpha]_D^{25} = -39.0°$.

D. Solubility: Malformin was readily soluble in acetic and formic acid, phenol, methyl cellosolve, and dimethyl formamide; sparingly soluble in ethyl acetate, ethanol, methanol, and acetone; difficultly soluble in water, benzene, petroleum ether (B.R. 60 to 70°), and chloroform.

E. Electrophoretic Mobility: Column electrophoresis was used under acidic and basic conditions. A rectangular, horizontal column (1.6 × 3 × 38 cm), consisting of 19 removable 2 cm sections, was packed with polyvinyl resin (Geon, B. F. Goodrich Chemical Co.) mixed with 0.1 N acetic acid buffer (pH 3.5), or 0.1 M phosphate buffer (pH 8.7) containing 10% methyl cellosolve. Excess buffer was removed from both ends of the column. Resin from the center section of the column was removed and replaced with resin previously mixed with 0.7 ml malformin solution containing 300 to 500 μg malformin and one mg glucose (control) in 1 ml of buffer. Electrophoresis followed for 17 hours (70 volts/cm, 2.5×10^{-3} amp/cm² under acidic conditions; 70 volts/cm, 5.7×10^{-3} amp/cm² under basic conditions). The resin from each section of the column was removed and washed with 4 ml of water. An aliquot of the eluate from each section was filtered, diluted, and tested for malformin by the corn root test. Malformin was also detected by mixing 1 ml of the eluate with 0.5 ml 15 N H_2SO_4 and hydrolyzing at 121° C for 17 hours. The hydrolysate was adjusted to pH 5.0 with 5 N NaOH, mixed with 2 ml 0.2 N citrate buffer (pH 5.0) and 1.5 ml KCN-ninhydrin solution to detect amino acids (see below). The solutions were heated at 100° C for 15 minutes. The control, glucose, was detected by the anthrone-H_2SO_4 reagent (8).

In acidic conditions malformin and glucose were detected only at the origin and weakly in the first section toward the negative pole. Identical results were obtained in basic solvent except glucose was detected only at the origin. The slight movement of malformin toward the negative pole under basic conditions was not considered significant. It was concluded that malformin was not amphoteric.

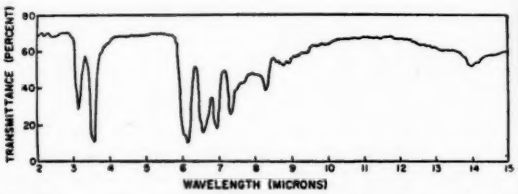


Fig. 2. Infrared spectrum of malformin in Nujol Mull.

IV. Hydrolysis of Malformin: Information derived from the elemental analysis, electrophoresis, and the movement of malformin in various solvents of the purification process led us to expect that malformin was a peptide with no free terminal groups. Malformin was hydrolyzed in 6 N HCl for 20 hours at 121° C. The hydrolyzate gave a strong positive reaction with ninhydrin, thus confirming its peptide nature. Paper and column chromatography were used to identify the amino acids and determine their molar ratio.

A. Paper Chromatography: Malformin (2 mg) was dissolved in 0.4 ml acetic acid and mixed with 2 ml of 6 N HCl. The solution was sealed in a small pyrex test tube and hydrolyzed for 20 hours at 121° C. The hydrolyzate was dried and 0.5 ml of water was used to dissolve the residue. Five μl were applied to strips of Whatman no. 1 paper. The amino acids were separated with the organic phase of a mixture of butanol, acetic acid, and water (50:1:50 v/v) using descending, multiple (two multiplications) chromatography. Four spots were detected with ninhydrin. Their Rf values were 0, 0.37, 0.57, and 0.62. By comparing these spots with those of authentic samples it was concluded that three of the amino acids were valine (Rf 0.37), isoleucine (Rf 0.57), and leucine (Rf 0.62).

The amino acid which remained at the origin was considered to be a sulfur-containing amino acid, and because of its low Rf value, possibly cystine. The intensity of this spot varied with the conditions of hydrolysis. It is known that cystine is not stable to the usual methods of hydrolysis, and peptides or proteins containing cystine are often oxidized by performic acid prior to hydrolysis (19). The oxidation product of cystine, cysteic acid, is stable during hydrolysis. Two mg of malformin were oxidized with performic acid for 4 hours at 4 to 5° C (performic acid was prepared by mixing 22.5 ml of formic acid and 2.5 ml of 30 % H_2O_2 at room temperature for 1 hr). After oxidation the solution was evaporated in vacuo, and the residue was hydrolyzed in 2 ml of 6 N HCl at 121° C for 17 hours. Descending chromatography, with methanol, water, pyridine (80:20:4 v/v) was used to separate the amino acids. Two spots, Rf 0.6 and 0.8, were detected with ninhydrin. The compound with an Rf value of 0.6 was identified as cysteic acid by comparing with authentic material. The other spot corresponded to a mixture of leucine, isoleucine, and valine.

Alkaline hydrolysis of malformin (2 mg) was done in 2 ml of 1 N $Ba(OH)_2$ at 121° C for 20 hours. The hydrolyzed material was brought to pH 5 with H_2SO_4. A precipitate of $BaSO_4$ was removed by centrifugation and washed twice with 3 ml aliquots of water. The hydrolyzate and the $BaSO_4$ washings were combined, evaporated to dryness, and dissolved in 0.5 ml of water. This solution (0.5 μl) was chromatographed using the butanol, acetic acid, water solvent. Four spots were detected with Rf values of 0.15, 0.37 (valine), 0.57 (isoleucine), and 0.62 (leucine). The new spot at Rf 0.15 was derived from cystine during alkaline hydrolysis as shown by similar experiments with authentic cystine.

Carbohydrates were excluded as components of the hydrolyzates by spraying the chromatograms with aniline hydrogen phthalate (16), α-aminophenol-phosphoric acid (10), and ammoniacal $AgNO_3$ (15).

The molar ratio of the component amino acids of malformin was determined by the paper chromatographic method of Kay, Harris, and Entenman (11). In this method the amino acids were detected with ninhydrin, eluted from the paper with alcohol, and measured photometrically. Oxidized and hydrolyzed malformin was chromatographed using butanol, acetic acid, and water as described. Because isoleucine and leucine were not completely separated, they were combined. The molar ratio of cysteic acid, valine, and the leucine-isoleucine mixture was 2:1:1.8, respectively.

B. Column Chromatography: To obtain further evidence for identifying the amino acid components of malformin and their molar ratios the methods of Moore and Stein were used (13) with slight modification. Dowex 50-X8W resin (200–400 mesh) was used to prepare a column (1.0 × 75 cm) with 0.1 N citrate buffer (pH 4.25). The temperature of the column was maintained at 50 ± 1° C by circulating water through the jacket of the column. Malformin (0.3–0.4 mg), hydrolyzed directly or after oxidation with performic acid, was applied to the column in 2.5 ml of 0.1 N citrate buffer (pH 3.42). The sample was washed onto the column with two 0.5 ml aliquots of the same buffer and eluted with 0.1 N citrate buffer (pH 4.25). Because the hydrolyzate contained only four amino acids only this buffer was used for elution. The fractions (1.7 ml) were collected at a constant rate of 5 ml per hour by regulating the position of the buffer reservoir and by air pressure. The amount of amino acid in each fraction was determined by the method of Yemm and Cocking (20) as follows: to each fraction 0.05 ml of 1 N NaOH, 1.5 ml of 0.2 N citrate buffer (pH 5.0), and 1.3 ml of KCN-ninhydrin solution were added. The solutions were heated at 100° C for 15 minutes and cooled in cold water. After adding 5 ml of 60 % ethanol to each fraction the color intensity was measured photometrically at 570 mμ. Known amino acids were used to prepare standard curves. The results of direct hydrolysis of malformin and hydrolysis after oxidation are given in figures 3 and 4. By comparing the position of the peaks with those of standard amino acids, the amino acids were identified as leucine, isoleucine, valine, and cystine (cysteic acid after oxidation). Separation of leucine and isoleucine was complete. The molar ratio (table II) was calculated from the total amino acid content under each curve. The value obtained for ½ cystine, when malformin was hydrolyzed directly, was low because this amino acid is unstable.

If the results of elemental analysis, molecular weight determination, and the paper chromatography are considered the molar ratio of leucine, isoleucine,

TABLE II

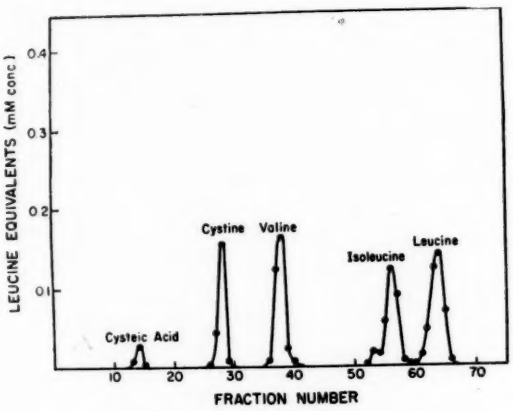
MOLAR RATIO OF AMINO ACID COMPONENTS OF MALFORMIN ISOLATED FROM A. NIGER 56–39

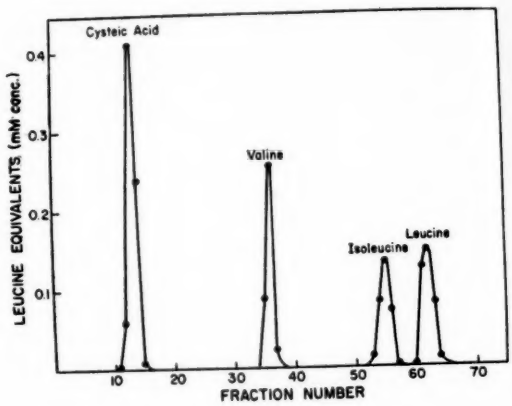
AMINO ACID	MALFORMIN	OXIDIZED MALFORMIN
Leucine	1.00 (1)*	1.00 (1)
Isoleucine	0.79 (1)	0.76 (1)
Valine	0.80 (1)	0.99 (1)
½ Cystine	1.04 (2)	0.00 (0)
Cysteic acid	0.08 (0)	1.97 (2)

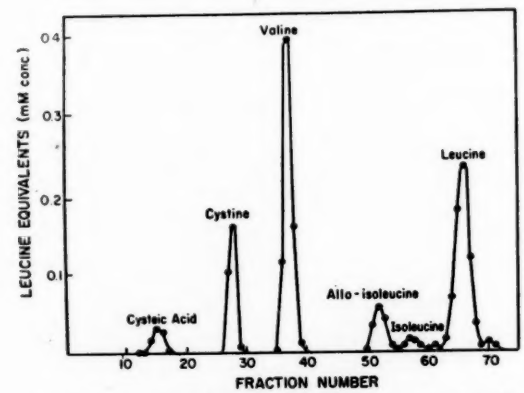
* The values in parentheses were calculated from the experimental formula of $C_{23}H_{41}N_5O_5S_2$.

valine, and ½ cystine must be 1:1:1:2, respectively. A cyclic peptide containing these amino acids in such a ratio has a molecular formula identical with that calculated from the results of elemental analysis. The molar ratio obtained by column chromatography showed slight deviations from the calculated ratio, especially the isoleucine value, but support the 1:1:1:2 ratio.

V. TEST FOR -SH GROUP IN MALFORMIN: The nitroprusside test of Anson (1) and the sodium azide-iodine test of Feigl (9) were used to detect -SH groups. No reaction occurred in either of these tests or after hydrolysis of malformin by a mixture of 90 % formic acid and 6 N HCl (1:1 v/v) in evacuated







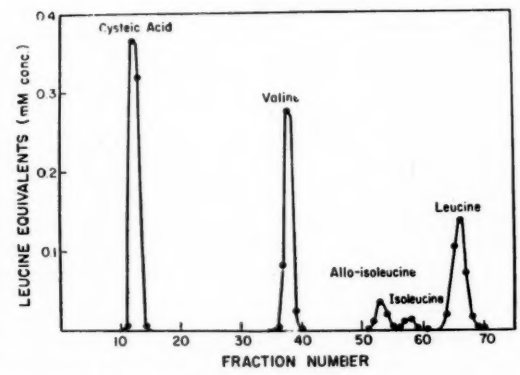


FIG. 3. (*upper left*). Amino acid components of malformin produced by *A. niger* 56–39. Samples hydrolyzed without prior oxidation.

FIG. 4. (*upper right*). Amino acid components of malformin produced by *A. niger* 56–39. Samples hydrolyzed after oxidation with perfomic acid.

FIG. 5. (*lower left*). Amino acid components of malformin produced by *A. niger* 56–30. Samples hydrolyzed without prior oxidation.

FIG. 6. (*lower right*). Amino acid components of malformin produced by *A. niger* 56–30. Samples hydrolyzed after oxidation with performic acid.

and sealed tubes. Under these conditions of hydrolysis, conversion of cysteine to cystine is limited. The lack of -SH groups indicated that, in the isolated malformin, the S-S bridge of cystine was present.

VI. Production of Malformin by Other Strains of A. niger: The yield of purified malformin from culture filtrates of 56–39 gradually decreased from a high of about 15 mg per liter to less than 1 mg per liter. Consequently, a screening program was initiated to obtain higher yielding strains. Strain 56-30 was selected. When grown on a corn steep-glucose medium, it yielded approximately 35 mg per liter of culture filtrate. Because the isolation method, infrared spectrum, and elemental analysis were identical, it was considered that malformin from 56–39 and 56-30 were identical. When hydrolyzates of malformin from 56–30 and 56–39 were compared, differences were found. On paper chromatograms, no differences could be detected in the relative intensity of the leucine, valine, and cystine (or cysteic acid) spots. However, the intensity of the isoleucine spot was much weaker for the amino acids from 56–30 than from 56–39, whether the malformin was hydrolyzed directly or after oxidation with performic acid. For more accuracy, the molar ratio of the component amino acids of malformin isolated from 56–30 was also investigated by the method of Moore and Stein (13). The amino acid curves obtained from malformin hydrolyzed directly (fig 5) and after oxidation (fig 6) indicated that the amino acid which was considered to be isoleucine, on the paper chromatograms, was actually allo-isoleucine. Furthermore, the molar ratio of this amino acid was low (table III). The molar ratio of the other amino acids was essentially the same as that obtained from 56–39. Apparently, the two malformins are different. Strangely, no differences were detected in elemental analysis or infrared spectrum. Clarification must await further experimentation.

Table III

Molar Ratio of Amino Acid Components of Malformin Isolated From A. Niger 56–30

Amino acid	Malformin	Oxidized malformin
Leucine	1.00	1.00
Isoleucine	0.07	0.09
Allo-isoleucine	0.22	0.22
Valine	1.05	1.07
½ Cystine	0.77	0.00
Cysteic acid	0.11	2.13

Malformin, isolated similarly from six other strains, was hydrolyzed and examined for amino acid content by paper chromatography. All strains produced malformin like that of 56–39. Only 56–30 produced the different malformin. The activity of these compounds on bean seedlings and corn roots appeared the same.

Discussion

Evidence obtained earlier (6) indicated that the compounds produced by *A. niger* which caused malformations on beans and curvatures on corn roots were identical. The activity of isolated malformin on both bean stems and corn roots confirmed the results obtained by paper chromatography.

The neutral nature of malformin was shown by: A: no movement by electrophoresis, B: solubility properties, C: no adsorption by anion and cation exchange resins, and D: negative ninhydrin test before hydrolysis. The amino acid components of malformin are all neutral. These facts suggest that malformin is probably a cyclic peptide.

Two formulae are proposed for malformin isolated from strain 56–39. The compounds differ only by two hydrogen atoms, depending on the presence of cystine or two cysteine residues in the molecule. Because no free -SH groups were detected, the first formula should be assigned to malformin. Under the conditions of isolation it seems likely that any free -SH groups present originally would be oxidized to form the S–S bridge. Actually, the activity of this compound may depend on the interconversion of the -SH and S–S forms. The necessity of these groups is emphasized by a complete loss of activity on beans when malformin was oxidized with performic acid to form $-SO_3H$ radicals. Many enzymes, antibiotics, toxins, hormones, and plant growth substances, are believed to be active because they contain or react with sulfhydryl groups (3, 12, 14). Roberts and Lucchese (18) and Pilet (17) have observed that -SH groups are in highest concentration in the apical meristems of plants. These regions respond most strongly to malformin.

Malformin contains an unusually high percentage of cystine (40 % calculated on the basis of ½ cystine). Malformin isolated from strain 56–30 contains an uncommon amino acid, allo-isoleucine, first isolated from a natural compound as a degradation product of actinomycin C (4).

Our results suggest that two malformins exist. The first, from strain 56–39 and other strains of *A. niger*, contains valine, leucine, isoleucine, and ½ cystine in a 1:1:1:2 ratio, respectively. The second malformin, from strain 56–30, appeared identical with respect to method of isolation, elemental analysis, and infrared spectrum. The ratio of valine, leucine, and ½ cystine of the second malformin was similar to that of the first but the ratio of isoleucine was low. Also, the second malformin contained allo-isoleucine. These differences must be resolved before a structure can be proposed. Newly isolated peptides often are later shown to be mixtures. This possibility must also be considered for malformin. We have shown the possibility of two kinds of malformin from strains 56–39 and 56–30. If malformin

from 56–39 is a mixture of the two malformins, the molar ratio for valine, leucine, and ½ cystine would be unchanged. However, because malformin from 56–30 has a low ratio of isoleucine, the isoleucine ratio of the mixture would be decreased. Our data support this possibility, in that the molar ratio of valine, leucine, and ½ cystine closely approximated 1:1:2, but the molar ratio of isoleucine was low (0.79).

Summary

Malformin was isolated from culture filtrates of the fungus, *Aspergillus niger* 56–39, and found to be a neutral peptide containing four amino acids, valine, leucine, isoleucine, and ½ cystine. The molar ratio obtained for these amino acids supported a 1:1:1:2 ratio, respectively. It was suggested that malformin is a cyclic peptide. The molecular formula, $C_{23}H_{39}N_5O_5S_2$, was proposed. The infrared spectrum showed strong adsorption bands at 3.01, 6.06, and 6.54 μ. A high malformin-producing strain, 56–30, produced malformin which differed from that produced by strain 56–39. This new malformin contained allo-isoleucine.

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STUDIES ON RESPONSE OF BEAN SEEDLINGS & CORN ROOTS TO MALFORMIN [1, 2, 3]

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In 1958 I reported that culture filtrates of the fungus, *Aspergillus niger*, induce curvatures and grotesque malformations on bean plants (1). Subsequently, it was shown that roots obtained from corn seeds germinated in the presence of *A. niger* culture filtrate also develop severe curvatures (2). Evidence obtained by paper chromatography, using a variety of solvents, indicated that the compounds causing bean stem malformations and corn root curvatures were identical. Furthermore, their Rf values were different from those obtained for indoleacetic acid (IAA). Histological examination of irregular stem enlargements on bean plants indicated that the malformations resulted from partial loss of polarity of the cells in the actively growing region of young internodes and petioles (6).

The name, malformin, has been proposed for the active compound produced by *A. niger* (3).

Recently, malformin was isolated from culture filtrates of *A. niger*, and found to be a neutral cyclic peptide (7). The availability of pure malformin has made it possible to conduct more quantitative experiments not possible with the crude material. These experiments have emphasized the qualitative and quantitative responses of bean stems and corn roots to malformin.

MATERIALS & METHODS

SOURCE OF MALFORMIN: Because of the low yield of malformin from the original isolate of *Aspergillus niger* van Tiegh. (56-39), other soil isolates were tested for their ability to produce malformin. A high-yielding strain, 56-30, was found and was used throughout these studies. Corn steep-glucose medium was employed as described in the earlier reports. The methods used to isolate malformin from *A. niger* culture filtrate are reported in the preceding paper. The final product was obtained after adsorption and elution from columns of activated alumina.

Stock solutions were obtained by dissolving 2 to 4 mg of malformin per ml of warm methyl cellosolve and diluting with 9 ml of water for each ml of methyl cellosolve used. These stock solutions were frozen with no apparent loss of activity.

In a few experiments, crude ether extracts of *A. niger* culture filtrate were used to treat plants as indicated in the text.

TREATMENT OF PLANTS: The method used to treat the young apical bud of bean seedlings (*Phaseolus vulgaris* L. var. Black Valentine) and their stage of growth at the time of treatment are essentially the same as reported earlier. However, the volume of solution applied was measured with an Agla micrometer syringe apparatus. In all cases, 0.001 ml of solution was placed on one side of the apical bud before elongation had begun and when the primary leaves were 1/4 to 1/3 of their fully expanded size. The solutions contained four drops of Tween 80/100 ml. The plants were grown in vermiculite, three to four plants per 4 inch pot, under ordinary greenhouse conditions. After treatment and on days of high sunlight intensity the plants were shaded with a single layer of cheese cloth. Usually, five to six randomized blocks of 12 to 15 plants were treated with each solution. The results from each block of 12 to 15 plants were averaged and considered as one replicate. Observations and measurements were made 8 to 12 days after treatment by tabulating the number of plants responding to malformin (stem and petiole irregularities, downward curvature of the first compound leaf) and measuring the length and the wet and dry weight of the first internode above the simple leaves.

Corn roots (*Zea mays* L. the single cross WF9 × 38–11) were treated in one of two ways. Using the filter paper method, 2.5 ml of the solutions were used to moisten Whatman no. 1 filter paper (9.0 cm) in petri dishes. The seeds were thoroughly washed in deionized water and 10 seeds were placed, embryo down, on the periphery of the filter paper. The seeds were arranged in sets of five on opposite sides of the dish and oriented so that the roots would grow toward one another. The plates were kept at 26 to 27° C for 3 days. The number of roots having at least a 90° curvature from the original direction of growth was then counted. Usually, 150 to 200 seeds were used for each treatment. The roots were removed from the seeds, divided into lots of 50, and used for wet and dry weight determinations. The average weight for each group of 50 was considered as a single replicate.

In a number of experiments corn roots were treated by placing 0.001 ml of the solution approximately one mm behind the root tip. This method, referred to as tip treatment, also employed the Agla micrometer

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syringe apparatus. The root tips were usually treated 40 to 48 hours after the seeds were arranged on filter paper as described above. In these tests 2.5 ml of double distilled water was used to moisten the papers. The roots were examined for curvatures 24 hours after treatment.

In evaluating the production of curvatures on corn roots by malformin I have distinguished two degrees of response. In the first, designated as 90° curvatures, *all* roots were tabulated that showed at least one 90° curvature from the original direction of growth. In the second, designated as severe curvatures, *only* those roots were tabulated which showed at least one continuous 360° curvature. Usually these severe curvatures were substantially greater than 360°, and the two degrees of response were easily differentiated. Illustrations of typical root curvatures are shown in figures 1 and 2. The roots tabulated as having 90° curvatures included, therefore, the roots having severe curvatures as well as the more moderate curvatures (90°–360°).

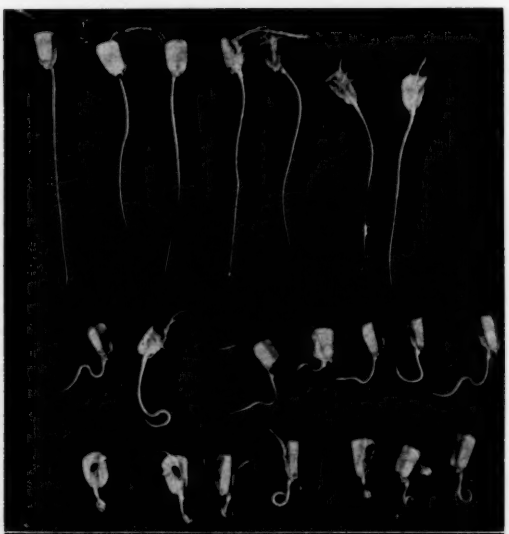

FIG. 1. (*upper*). Corn root curvatures induced by malformin. Control (*top row*), moderate curvatures (*middle row*) and severe curvatures (*bottom row*).

FIG. 2. (*lower*). Three types of severe curvatures induced by malformin on corn roots. These curvatures are usually referred to as "figure eight" (*left*), "corkscrew" (*middle*), and "*knotted*" (*right*).

RESULTS

RESPONSE OF BEAN PLANTS TO MALFORMIN: The percentage of plants which responded to malformin treatment, as determined visually, is shown in figure 3. Malformin has been detected when as little as 1×10^{-4} μg per plant was applied. Although the percentage of plants responding to this dosage was small, the nature of the response is such that it is easily detected. Because of the low solubility of malformin, quantities of more than 1×10^{-1} μg were not employed. The solvent, methyl cellosolve, was tested in the same concentrations as the malformin solutions and was without effect.

The height of the first internode above the simple leaves was measured to the nearest mm. This internode was chosen because it appeared to be most strongly affected. Due to the great variation in the growth of the plants the values are reported (fig 4) as percentages of the height of the control plants (water treated). No significant (5% level) growth inhibition could be detected using 1×10^{-4} μg per plant but visual observations showed that a small percentage of the plants responded. Thus, visual inspection will more readily detect a response at this low dosage. Preliminary evidence indicated that IAA, applied in varying concentrations at daily intervals after malformin treatment, had no effect on the production of malformations by malformin nor did it stimulate the growth of the internodes. Similar evidence indicated that, although gibberellic acid stimulated significantly the growth of the malformin inhibited internodes, the final height remained considerably below that of the controls receiving neither malformin nor gibberellic acid. The stimulation caused by gibberellic acid may be misleading since even severely malformed internodes often have regions which appear normal. These regions, not apparently affected by malformin, may be responding to gibberellic acid. Plants treated with both malformin and gibberellic acid showed no apparent reduction in the severity of the malformations.

Wet and dry weights of the first internode above the simple leaves are given in table I. Neither differ-

TABLE I

WET AND DRY WEIGHTS OF FIRST INTERNODE ABOVE SIMPLE LEAVES OF BEAN PLANTS TREATED WITH MALFORMIN*

μg MALFORMIN/PLANT	WT/INTERNODE	
	WET (Gm)	DRY (Mg)
0 (Control)	0.15	19
10^{-4}	0.15	19
10^{-3}	0.16	19
10^{-2}	0.16	17
10^{-1}	0.16	18

* Average of three experiments.

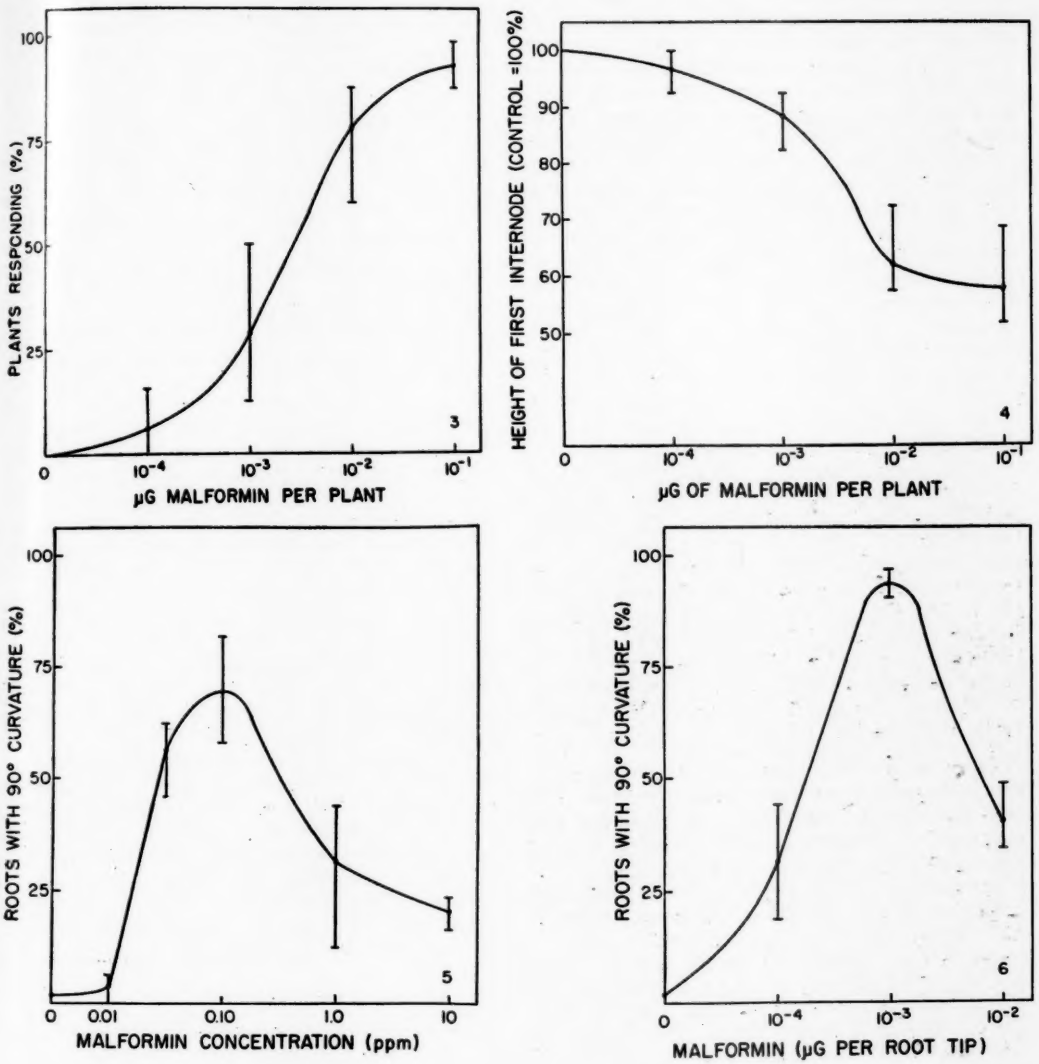

FIG. 3. Response of bean plants to malformin as determined by visual inspection.

FIG. 4. Inhibition of first internode above simple leaves of bean plants by malformin.

FIG. 5. Production of corn root curvatures by malformin. Seeds were germinated on filter paper moistened with the solutions.

FIG. 6. Production of corn root curvatures by malformin. Root tips were treated with the solutions and evaluated for curvatures after 24 hours.

ed significantly from the control plants. This was surprising because the internodes of severely affected plants were approximately half the height of the control plants, although the internodes of the treated plants were considerably thicker. These results support the belief that malformin has an effect more subtle than merely affecting cell division or enlargement (6). A study of sections taken from treated plants indicated that this compound seemed to alter the polarity or orientation of the developing cells.

In a few tests ether extracts of culture filtrates were employed at two times the original concentration. In the first experiment bean seedlings (7–8 days old) were gently removed from vermiculite and transferred to beakers containing the test solutions. None of the solution reached the above ground portion of the plants. After 24 hours the seedlings were transferred to beakers containing water. Observations were made at the end of 8 to 11 days. Malformin was translocated through the roots to the apex. In this case, however, the first internode above the simple leaves showed little to no effect of the treatment. The second and third internodes showed most strongly the malformations characteristic of malformin treatment. In a second test bean seeds were soaked overnight in the test solutions and then planted in vermiculite. No effect was noted up to 2 weeks after planting. An attempt was made to treat the seedlings through the leaves. Preliminary evidence had indicated that this method was ineffective (1). In these experiments, however, the tips of the simple leaves were removed and the leaf immersed in a vial of the test solution for 48 hours. No effect was observed on the plants. Of the three methods only root uptake was effective.

Response of Corn Roots to Malformin: Although the roots respond to malformin no response has been obtained on the above ground portions of corn plants. Corn seedlings have been injected, sprayed, and the roots immersed in malformin solution for 24 hours, but only a slight growth inhibition at high concentrations has been found. Experiments, using the filter paper method, were carried out to evaluate the response of corn roots to malformin. The percentage of roots which showed at least a 90° curvature at various concentrations of malformin is shown in figure 5. The small percentage of 90° curvatures at 0.01 ppm was not significant (5 % level). The optimum concentration of malformin for 90° curvatures was 0.1 ppm. Higher concentrations decreased the response. Since it was necessary to dissolve malformin in a small quantity of methyl cellosolve before making the dilutions, the decline in curvatures at higher malformin concentration might be due to the higher concentration of methyl cellosolve. Using the optimum concentration of malformin for curvature (0.1 ppm) the methyl cellosolve concentration was increased to the levels employed at 1.0 and 10 ppm malformin to determine whether or not the percentage of roots responding decreased. Methyl cellosolve did decrease the root curvatures, but this does not prove that the same phenomenon is reducing the malformin response at 1.0 and 10 ppm malformin. The same decline in activity occurred at 1.0 and 10 ppm when acetic acid and phenol were used to dissolve the malformin; it also occurred with ether extracts of the culture filtrate or the crude filtrate itself. In the same manner in which it was shown that methyl cellosolve decreased root curvatures (above) similar tests have shown that when acetic acid is used as a solvent the decline in root curvatures at 1.0 and 10 ppm was not caused by the higher concentration of acetic acid. For these reasons it is believed that the curve shown in figure 5 is a correct representation.

The highest percentage of roots responding to purified malformin in eight experiments was 82.6 (average = 70.3). However, using crude ether extracts of *A. niger* culture filtrate, in 12 of 14 tests the average percent response was 94.0. When the method of treatment was changed the purified material gave results comparable to those obtained when crude ether extracts were used (see below).

Although malformin had little or no effect on the wet and dry weight of bean internodes, it inhibited both the wet and dry weight production of corn roots (table II). In each of the experiments the solvent used to dissolve malformin, either methyl cellosolve or acetic acid, was tested by itself in concentrations corresponding to its level in the various malformin solutions. Any change in the wet or dry weight caused by the solvent was used as a correction factor for the wet and dry weights of corn roots treated with malformin. It was assumed that the effect of the solvent itself was not changed in the presence of malformin, although no information was obtained to substantiate this assumption.

The influence of only one variable on the production of corn root curvatures was investigated. If the volume of solution used to moisten the filter paper was increased over 2.5 ml the percentage of roots with 90° curvatures decreased (table III). The reason is not clear since roots obtained using the larger volumes appeared healthy and vigorous and were not apparently suffering from a lack of sufficient aeration.

To locate the region of the root responding to mal-

Table II

Effect of Malformin on Wet and Dry Weights of Corn Roots

Malformin (ppm)	Wt/root (% of control)	
	Wet	Dry
0 (Control)	...	...
0.001	106.0±6.2*	106.0±7.3
0.01	104.0±5.6	101.0±4.6
0.1	80.7±4.2	91.2±2.7
1.0	81.3±7.3	92.3±4.6
10.0	80.7±6.5	85.7±5.0

* Confidence intervals (5 % level)

TABLE III

EFFECT OF SOLUTION VOLUME ON ROOT CURVATURES INDUCED BY MALFORMIN*

ml MALFORMIN/PLATE (0.1 ppm)	90° ROOT CURVATURES (% OF CONTROL)
2.5 (Control)	...
3.0	86.4
3.5	57.1
4.0	52.0
4.5	33.1
5.0	34.9

* Average of four experiments.

formin, roots were treated (2 days after being placed on filter paper moistened with water) at three positions with 0.001 ml of the malformin solutions. These positions were the tip or 1 to 2 mm behind the tip, the middle, and as close as possible to the seed. Curvatures resulted only when the tip was treated. Unless penetration influenced the results, it appears that, as with the bean stems, the actively dividing cells are most seriously influenced by malformin. The dosage-response curve is shown in figure 6. The curve is similar to that obtained by the filter paper method. The optimum dosage was 1×10^{-3} µg per root while 1×10^{-4} µg could be detected. Two observations should be made regarding the tip method of treatment: A: The highest percentage of 90° curvatures obtained by this method was higher than when the filter paper method was used. Thus, in each of three experiments using the root tip treatment, the percentage of roots responding at the optimum concentration was over 90 % (average = 93.6) and was comparable to that obtained when crude extracts were used. B: The percentage of severe curvatures was greatly increased when the root tip treatment was employed. The results of simultaneous experiments using the two methods are given in table IV.

The only variable investigated using the tip treatment was the influence of the age of the roots at the time of treatment on their response to malformin. Only the severe curvatures were tabulated, because there appeared to be little effect on the production of 90° curvatures in the age groups used. The age of the roots was taken as the number of hours after the seeds were washed prior to being placed on filter papers moistened with 2.5 ml of water. For each of the three time intervals the roots were treated with 1×10^{-3} µg per root. Curvatures were determined 24 to 30 hours after each treatment. The results (table V) indicate that roots treated at 38 and 47 hours differ little in their response to malformin. However, there was a sharp decline in severe curvatures when roots were treated after 55 hours.

TABLE IV

PRODUCTION OF CORN ROOT CURVATURES BY MALFORMIN USING 2 METHODS OF TREATMENT*

METHOD OF TREATMENT	% ROOT CURVATURES	
	90°	SEVERE
A. Filter paper (ppm)		
Control (water)	2.0	0
Malformin (1.0)	37.1	4.1
" (0.1)	75.7	2.0
" (0.01)	3.0	0
B. Tip treatment (µg/root)		
Malformin (10^{-3})	92.5	32.8
" (10^{-4})	24.4	6.6

* Average of two experiments.

TABLE V

EFFECT OF ROOT AGE ON PRODUCTION OF SEVERE CURVATURES BY MALFORMIN*

AGE OF ROOTS (hr)	% SEVERE CURVATURES
38	28.9
47	27.7
55	5.5

* Average of two experiments.

EFFECT OF MALFORMIN ON OTHER PLANTS: A small survey was conducted to determine if malformin was active on plants other than those used in the above studies. Malformin was active on the above ground portions of a variety of dicotyledonous plants (table VI). In the case of these treatments, ether extracts of the culture filtrates were used to treat the apical buds of the seedlings. The extracts were taken up in water containing four drops of Tween 80/100 ml to give a final concentration of two times that of the unextracted filtrate. The percentage of plants which responded for a given species was usually more than 50 percent. As indicated earlier, attempts to obtain curvatures or malformations on the above ground portions of corn plants failed. Eight other species of monocots were treated with malformin (table VI). Only Sudan grass and onion responded to the treatment. On Sudan grass the stem curvatures occurred at the base of the coleoptile and resembled the corkscrew curvatures on corn roots. Although Sudan grass and onion responded, the percentage was small (less than 5 %). This was in contrast with the results obtained by treating various dicotyledons.

A series of preliminary experiments was done in an attempt to induce root curvatures on dicotyledonous plants. Ten species were tested but none responded to malformin (bean, mustard, tomato, pumpkin, radish, cucumber, muskmelon, squash, collard, carrot). The varieties were the same as those listed in table VI. Root curvatures were obtained on one monocot other than corn (barley) but not on the other monocots listed (table VI).

TABLE VI

RESPONSE OF VARIOUS PLANTS TO MALFORMIN*

NAME OF PLANT	STEM CURVATURE	STEM ENLARGEMENT
Lycopersicon esculentum Mill. (Tomato var. Kokomo)	+	+
Cucumis melo L. (Muskmelon var. Pride of Wisconsin)	+	+
Cucumis sativus L. (Cucumber var. Davis Perfect)	+	+
Glycine Max (L.) Merr. (Soybean var. Hawkeye)	+	+
Vigna sinensis (Torner) Savi (Cowpea var. Blackeye)	+	+
Raphanus sativus L. (Radish var. Scarlet Globe)	−	−
Cucurbita pepo L. (Pumpkin var. Winter Queen)	+	+
Cucurbita pepo L. var. Melopepo Alef. (Squash var. Uconn)	+	+
Phaseolus oleracea L. (Lima Bean var. Triumph)	+	+
Daucus carota L. var. Sativa DC (Carrot var. Touchon)	+	+
Capsicum fruitescens L. (Pepper var. Large Bell)	+	+
Brassica botrytis L. (Broccoli var. De Cicco)	+	+
Brassica oleraceae L. var. capitata L. (Cabbage var. Red Acre)	−	−
Brassica pervidis Bailey (Mustard var. Tendergreen)	+	−
Brassica oleracea L. var. acephala DC. (Collard var. Georgia)	−	−
Citrullus vulgaris Schrad. (Watermelon var. Rhode Island Red)	+	+
Beta vulgaris L. (Beet var. Fireball)	+	+
Centaurea cyanus L. (Centaurea var. Red Boy)	−	−
Zinnia elegans Jacq. (Zinnia var. Blaze)	−	+
Ricinus communis L. (Castor Bean var. Crimson Spire)	+	+
Cosmos bipinnatus Cav. (Cosmos var. Sensation Dazzler)	+	+
Tagetes erecta L. (Marigold var. Early Sunset)	+	+
Solanum melongena L. var. esculentum (Egg Plant var. Black Beauty)	+	?
Antirrhinum majus L. (Snapdragon var. Alaska)	−	?
Ipomoca purpurea Lam. (Morning Glory var. Candy Pink)	−	−
Impatients holstii Engler & Warb.	+	+
Callistephus chinensis Nees (Aster var. W. R. Super Giant)	−	−
Delphinium chelianthum L. (Delphinium var. Pacific Giant)	−	−
Poa pratensis L.** (Kentucky Blue Grass)	−	−
Bromus inermis Leyss.** (Brome Grass var. Lancaster)	−	−
Sorghum vulgare Pers.** (Sorghum var. RS610)	−	−
Sorghum vulgare Pers. var. sudanense** (Sudan Grass)	+	−
Allium cepa L.** (Onion, mixed var.)	+	−
Triticum aestivum L.** (Wheat var. Knox)	−	−
Hordeum vulgare L.** (Barley var. CI9574)	−	−
Avena sativa L.** (Oats var. Benton)	−	−
Zea mays L.** (Corn var. WF9 × 38-11)	−	−

* Plants treated with ether extract of *A. niger* culture filtrate at two times the normal concentration by placing two drops of solution as close to shoot apex as possible. Experiments in which a positive response was obtained the first time were not repeated. Experiments in which no response was obtained were repeated a second time.

** Plants treated by daily watering with solutions of purified malformin (5, 1, 0.1 ppm).

DISCUSSION

The results indicate that malformin has a high level of activity. In a comparison of various bioassays for auxins, Nitsch and Nitsch (4) pointed out that the minimum detectable amount of IAA in the Avena curvature test is 1.5×10^{-3} μg and 3×10^{-4} μg in the deseeded Avena test. Phinney and West reported that in the dwarf corn test for gibberellin 1×10^{-3} μg per plant induced a slight stimulation in growth (5). Malformin can be detected using either the corn root test or the bean test when 1×10^{-4} μg per plant is applied, placing it in the minimum detectable range of the Avena curvature tests.

Malformin has been detected in both the corn root test and the bean test using 1×10^{-4} μg per plant, but the optimum activity for each of these tests is quite different. In the corn root test 1×10^{-3} μg per root gives the optimum response, whereas the bean test approximately 1×10^{-1} μg per plant is optimum. These results parallel those of various auxin tests in which it has been shown that roots are generally more sensitive. One reason for the somewhat lower activity of malformin on beans may lie in the inaccessibility of the apical growing point. Since the meristematic region is surrounded by the leaf primordia it is necessary to apply the solution somewhat below the growing point, whereas roots have no such protective covering.

In a comparison of the two methods used to treat corn roots with malformin the tip treatment, although more time consuming, has several advantages. At optimum concentration the percentage of roots having 90° curvatures is higher using the tip treatment than when the seeds are germinated on filter paper moistened with the solution. Another advantage is the higher production of severe curvatures when the tip treatment is used, and the volume of solution required is considerably less. The advantages of using corn roots rather than beans are numerous. The environment can be more carefully controlled and no greenhouse space is required. Also, the corn root test is the more rapid of the two methods. If the tips of corn roots are treated the results can be obtained in 24

hours, whereas at least 4 days are necessary to determine a response on beans.

The above ground portions of a wide variety of dicotyledonous plants are sensitive to malformin (approx 71 % of the species tested). All efforts to produce curvatures on the roots of dicotyledonous plants have failed. On the other hand, although the roots of corn respond strongly to malformin, no curvatures or malformations have been detected on the above ground portions. Only two of nine species of monocots were affected by malformin and even when an effect was obtained, the percentage of plants responding was small. Thus, malformin appears to be most active on the above ground portions of dicotyledons but on the roots of monocotyledons (corn & barley). It should be pointed out that the attempts to demonstrate malformin activity on the roots of dicotyledons and on the above ground portions of monocotyledons have not been exhaustive. Further studies are needed to clarify this apparent unusual and differential response of monocotyledons and dicotyledons to malformin.

Summary

A quantitative study has been made of the response of beans (*Phaseolus vulgaris* L.) and corn (*Zea mays* L.) to purified malformin, a metabolic product of the fungus, *Aspergillus niger* van Tiegh.

I. On bean stems malformin produced a visible response when as little as 1×10^{-4} μg per plant was applied. The optimum response requires approximately 1×10^{-1} μg per plant. Neither the wet nor dry weights of malformin treated plants differed significantly from the controls. Malformin was translocated through the roots of bean plants to the region of the apical meristem. Seed soaking and leaf treatment were not effective methods of treatment of malformin on beans.

II. When various regions of corn roots were treated with malformin, curvatures were obtained only when the tips were treated. The optimum response was obtained using 1×10^{-3} μg per root tip. Malformin was detected by this method when as little as 1×10^{-4} μg was applied to the root tips. When corn seeds were germinated on filter paper moistened with solutions of malformin optimum curvatures were obtained at a concentration of 0.1 ppm. In both methods of treatment the percentage of roots responding decreased if the concentration of malformin was raised above the optimum.

III. Malformin was active on the above ground portions of 20 out of 28 species of dicotyledons. On monocotyledons, only onion and Sudangrass responded out of nine species tested. Attempts to show a response on the roots of dicotyledons failed.

Acknowledgments

I wish to thank Hikmet Egilmez for technical assistance.

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CYCLIC AND NON-CYCLIC PHOTOPHOSPHORYLATION IN CHLOROPLASTS DISTINGUISHED BY USE OF LABELED OXYGEN[1,2]

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Since it was first reported that isolated chloroplasts utilize light energy in the formation of ATP[5] (2) a number of catalysts of the reaction have been described (3). It was thought that these catalysts functioned as electron carriers in a cyclic process which does not involve molecular oxygen. The proposed process consisted of their reduction by a photochemically produced reductant and their subsequent reoxidation, in a series of phosphorylation linked reactions, by the associated photochemically produced oxidant (1). Phosphorylation was attributed to the reoxidation of the reduced catalyst on the basis of an analogy to oxidative phosphorylation in mitochondria. The non-participation of molecular oxygen in the cycle was postulated partly on the basis of an analogy to photophosphorylation in the chromatophores of Rhodospirillum (9) and partly on the basis of two experimental observations. These observations were A: the fact that phosphorylation by chloroplasts was independent of exogenous oxygen, especially at high concentrations of catalysts such as FMN (21), and B: the fact that experiments with labeled oxygen revealed none of the exchange which would result from its continuous production and consumption (14).

When it was discovered that phosphorylation also could be coupled to non-cyclic reactions such as the photochemical reduction of ferricyanide and TPN^+ by chloroplasts (3, 4) a re-evaluation of the earlier concept of cyclic phosphorylation became necessary. In the absence of any reoxidation process phosphorylation still occurred and consequently the site of phosphorylation (if only one) had to be assigned to the reduction process. Moreover, for a number of reasons enumerated below, the apparently settled matter of the non-participation of molecular oxygen in the FMN catalyzed system had to be reconsidered:

I. It had been shown that FMN is reduced by illuminated chloroplasts with the concomitant production of oxygen (10, 19). It seemed odd that the phosphorylation intermediates, phosphate and ADP, which stimulate rather than inhibit oxygen production in the presence of ferricyanide, should have abolished oxygen production with FMN.

II. Photophosphorylation with FMN was dependent on oxygen pressures at lower concentrations of the catalyst (17).

III. One of us (N.E.G.) showed that FMN reduction, whether measured spectrophotometrically in the absence of oxygen or indirectly by the method of Good and Hill (10), could be stoichiometrically related to ATP formation, the yield of ATP per mole of electrons transferred being the same as that observed in the reduction of ferricyanide (unpubl). This identity of stoichiometry seemed to preclude any unaccounted-for electron flux such as that involved in the catalysis of a cyclic process.

IV. Finally, an examination of the data obtained from the preliminary experiments with oxygen isotopes (14) revealed that the system used was probably diffusion limited. Because of a rather high rate of net oxygen uptake, caused presumably by the riboflavin sensitized photooxidation of ascorbic acid, the liquid phase oxygen concentration was vanishingly low. Since detection of an exchange reaction depends on the simultaneous movement of one isotope into the liquid and a different isotope out of the liquid, it is obvious that a diffusion limited system with its one way oxygen movement gives no information on exchange.

Meanwhile Jagendorf and his associates (13) and Hill and Walker (11) had shown that N-methylphenazonium ions (phenazine methosulfate or PMS) or pyocyanine catalyzed high rates of ATP formation by illuminated chloroplasts. Jagendorf was also able to show that PMS catalyzed phosphorylation was very little affected by 3-(4-chlorophenyl)-1,1-dimethylurea (CMU), a substance which inhibits both photosynthesis and the Hill reaction probably by inhibiting the mechanism of oxygen production (20, 8); photophosphorylation in the presence of ferricyanide or FMN was, on the other hand, completely inhibited by CMU.

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[5] The following abbreviations and trivial names have been employed: ADP, adenosine diphosphate; ATP, adenosine triphosphate; TPN^+, triphosphopyridine nucleotide; FMN, flavin mononucleotide; menadione, 2-methyl-1, 4-naphtho-quinone; CMU, 3-(4-chlorophenyl)-1, 1-dimethylurea; PMS (Phenazine methosulfate), N-methylphenazonium ion; pyocyanine, 1-hydroxy-5-methylphenazonium ion.

Moreover, earlier unpublished work by Hill, Davenport, and Good had suggested strongly that although pyocyanine did not undergo reduction by chloroplasts and reoxidation by molecular oxygen (10) it was nevertheless photochemically reduced by chloroplasts as judged by its catalysis of methaemoglobin reduction. Consequently the concept of a cyclic process not involving oxygen, which was rapidly becoming untenable with regard to the FMN system for which it was originally proposed, seemed to fit the pyocyanine and PMS systems very well.

To differentiate between these two mechanisms, that is to differentiate "dye" reduction followed by its reoxidation by molecular oxygen from dye reduction followed by reoxidation by some oxidized precursor of oxygen, the oxygen isotope exchanges in the various systems were investigated by means of the recording mass spectrometer described elsewhere (6, 12). In this report full appreciation has been accorded to the disequilibria associated with diffusion barriers. All isotope ratios, and consequently the calculated exchange rates, have been corrected for the effects of diffusion barriers on the isotope ratio of dissolved oxygen by the method of Brown and Weis (7). To keep such corrections small, the measured rates were kept as low as practicable by the use of minimal amounts of chloroplasts and the experimental vessel was shaken vigorously to minimize disequilibria across the gas-liquid interface. Moreover, the large net uptake of oxygen which characterized the earlier experiment (14) was almost completely abolished, even in the presence of FMN and ascorbate, by interposing an FMN filter between the light source and the reaction vessel. Under these revised conditions the amounts of ATP formation, oxygen production, and oxygen consumption were determined with FMN, menadione, pyocyanine, and PMS as catalysts.

Methods

Chloroplasts were obtained from market spinach. The leaves were ground in a chilled mortar with ice-cold buffer (0.35 M sucrose, 0.01 M NaCl, 0.05 M tris (hydroxymethyl) aminomethane, pH adjusted to 7.8 with H_2SO_4). The larger leaf fragments were removed by passing the brei through glass wool and smaller fragments were removed by centrifuging for 1 minute at 200 × *g*. The supernatant was then spun at 1000 × *g* for 7 minutes. The chloroplasts were resuspended and washed once with buffer. They were finally resuspended in buffer, filtered through glass wool to remove any clumps and stored briefly in an ice bath.

The reaction mixture consisted of chloroplasts containing from 30 to 155 μg chlorophyll, buffer, radioactive K_2HPO_4 (0.01 M), either ADP (10 μmoles) or ATP (1.0 μmoles) with hexokinase (0.6 mg) and glucose (30 μmoles), and $MgSO_4$ (24 μmoles). The various catalysts were added in the amounts indicated in the tables. The total volume was 3.0 ml. It was necessary to add catalase (highly purified from horse liver) to the PMS system in order to hasten the decomposition of hydrogen peroxide formed in the dark by PMS catalyzed oxidations of substances present in the chloroplast preparations.

Reactions were run at 14° C in a 15.8 ml rectangular vessel attached to the leak inlet to the mass spectrometer. The shaking rate was *236* oscillations per minute at a vessel excursion of *2* cm. Vessels were flushed with helium in darkness while they were being shaken. The oxygen labeled with a very large excess of O^{18} was admitted and the vessel was closed. Shaking was continued in the dark until the gas and liquid phases had completely equilibrated. At this point the light was turned on. About 15 minutes elapsed between the mixing of the reaction components and the beginning of illumination. An 18 minute light period was usually employed during which time the changing levels of oxygen isotopes were measured. After each run the mass spectrometer was calibrated using gas with a known oxygen concentration. In most experiments the very intense light was filtered through a concentrated solution of FMN before it impinged on the reaction vessel.

Following the period of illumination the reaction mixture was added to 10 % perchloric acid and the organic phosphate (ATP or glucose-6-phosphate) was measured as the radioactivity remaining after extraction of the orthophosphate as phosphomolybdate (18).

Results & Discussion

These results support unambiguously the reasoning outlined in the introduction: Phosphorylation with FMN was associated with the production and consumption of the expected amount of oxygen (table I). On this point our data agree with similar data of Nakamoto et al (16). On the other hand, pyocyanine and PMS catalyze a mechanism of phosphorylation in which molecular oxygen seems not to be involved (table II). Thus there is no longer any reason for retaining the original concept of cyclic phosphorylation with FMN since molecular oxygen is certainly involved in the cycle. The apparent oxygen independence of systems with high FMN concentrations is not really at variance with these findings since these concentrations of FMN would allow a fairly high concentration of $FMNH_2$ to accumulate without depleting FMN to a suboptimal concentration. This high concentration of $FMNH_2$ would react so promptly with the oxygen produced in situ that very little could escape to the gas phase. Consequently a closed system could be maintained indefinitely under a nitrogen atmosphere. At low concentrations of FMN, however, it would be impossible to build up enough $FMNH_2$ to trap all of the produced oxygen. Some oxygen would escape, more FMN would be reduced, and soon the remaining level of electron acceptor would be sub-optimal, resulting in severely limited electron transport and a correspondingly limited phosphorylation. It also seems unlikely that phosphorylation accompanies the reoxidation of $FMNH_2$

TABLE I

NON-CYCLIC PHOTOPHOSPHORYLATION*

CATALYST	CHLOROPHYLL CONC μg/REACTION	OXYGEN CONC %	OXYGEN EXCHANGE μATOMS		ATP FORMED μMOLES
			UPTAKE	PRODUCTION	
FMN (3.3×10^{-5} M)	31	0.87	1.75	1.7	1.85
"	85	0.28	2.7	2.6	2.6
Menadione (1.5×10^{-4} M)	40	0.45	2.45	1.65	0.57
"	42	0.32	1.25	0.72	0.33
"	42	0.35	1.00	0.70	0.31
FMN (3.3×10^{-5} M) + Ascorbate (3.3×10^{-3} M)	56	0.48	0.55	0.44	0.40
FMN (3.3×10^{-5} M) Menadione (1.0×10^{-5} M)	54	0.42	0.29	0.24	0.07
Ascorbate (3.3×10^{-3} M)	54	0.21	0.40	0.17	0.14

* The reaction mixture consisted of spinach chloroplasts, catalyst, tris buffer pH 7.8, radioactive K_2HPO_4 (0.01 M), ADP (10 μmoles) and $MgSO_4$ (24 μmoles), total volume 3.0 ml. Temperature 14° C. Light from a 1,000 w incandescent projection lamp was filtered through about two centimeters of a strong FMN solution. Time of illumination 18 to 37.8 minutes. The vessel was shaken at 236 cycles per minute with an excursion of 20 mm. Oxygen concentration is that of the liquid phase and is referred to the concentration in equilibrium with pure oxygen gas.

since this reoxidation is probably a spontaneous, non-enzymatic process. The accumulated evidence therefore suggests that phosphorylation with FMN as electron acceptor is in no way different from the process with ferricyanide as acceptor, save that in the reduced form of FMN is autoxidizable. Rates of electron transport, sensitivity to inhibitors, and the efficiency of phosphorylation in terms of the electron flux apparently are the same with either acceptor.

Photophosphorylation with menadione as electron acceptor has not yet been investigated thoroughly but the reactions catalyzed by this quinone probably are very similar to those catalyzed by FMN. However, menadione reduction in our experiments was not efficiently coupled to phosphorylation; oxygen production was greater than would have been predicted from the amount of ATP formed. This may be, in varying degrees, a property common to quinoid oxidants since the reduction of indophenol dyes by chloroplasts is completely dissociated from phosphorylation (15).

It is obvious (table II) that the methylphenazonium ion (PMS) and pyocyanine catalyze a reaction which does not involve the production and reutilization of molecular oxygen. From 5 to 15 μmoles of ATP were formed for each μatom of oxygen produced. Moreover, it has long been known that illuminated spinach chloroplasts in the absence of exogenous electron carriers exchange the oxygen of water with mo-

TABLE II

CYCLIC PHOTOPHOSPHORYLATION*

CATALYST	CHLOROPHYLL CONC μg/REACTION	OXYGEN CONC %	OXYGEN EXCHANGE μATOMS		ATP FORMED μMOLES
			UPTAKE	PRODUCTION	
N-methylphenazonium ion (PMS)	155	0.31	0.31	0.34	4.6
(3.3×10^{-5} M)	56	0.56	0.22	0.25	1.45
Pyocyanine	56	0.53	0.10	0.15	1.75
(3.3×10^{-5} M)	83	0.54	0.20	0.18	1.08
	66	0.52	0.16	0.13	1.55
	155	0.73	0.49	0.49	4.2

* The reaction mixture consisted of spinach chloroplasts, catalyst, tris buffer pH 7.8, radioactive K_2HPO_4 (0.01 M), ATP (1.0 μmole), hexokinase Sigma type II (0.6 mg), glucose (30 μmoles) and $MgSO_4$ (24 μmoles). Horse liver catalase was added with PMS. Total volume 3.0 ml. Temperature 14° C. Incandescent (white) light was used. Time of illumination 18 to 37.8 minutes, vessel shaken as in table I. Oxygen concentration expressed as above.

lecular oxygen at a slow rate (5) and consequently even the small amount of exchange observed in the presence of PMS and pyocyanine may be unrelated to their catalytic function.

The exclusion of molecular oxygen from the PMS and pyocyanine catalyzed electron transport chains is of some importance in fixing the site of CMU action. Bishop concluded that the phenyl-dimethylureas inhibit the mechanism which produces molecular oxygen because they inhibit photosynthesis but not photoreduction in Scenedesmus (8). Our results, coupled with the observations of Jagendorf cited above, provide further evidence correlating oxygen production and CMU sensitivity. Our evidence, in this case from in vitro studies, lends further support to Bishop's hypothesis.

Summary

Oxygen isotope exchanges during photophosphorylation by spinach chloroplasts have been followed with a mass-spectrometer. Oxygen exchange is equivalent to or greater than phosphate uptake when FMN or menadione is used as the catalyst if one assumes a P/O ratio of 1.0. With PMS or pyocyanine as the catalyst, phosphate uptake is from 5 to 15 times as great as the oxygen exchange. It is concluded that FMN and menadione catalyze a non-cyclic phosphorylation in which molecular oxygen is the final electron acceptor and that PMS and pyocyanine catalyze a cyclic phosphorylation in which molecular oxygen plays no part.

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NATURALLY OCCURRING GROWTH SUBSTANCES. II. AN IMPROVED STRAIGHT GROWTH TEST & ITS APPLICATIONS[1]

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The sustained interest of many investigators in the chemistry and biology of plant growth stimulants has resulted in the development of a variety of bioassay methods for the qualitative detection and quantitative estimation of these substances, both synthetic and of natural occurrence. Among the most common techniques based on differential responses are the Avena curvature test (13) and the slit pea stem test (14). Common straight growth assays include the Avena section test (2), the pea section test (4), and several methods which employ root tissue (1, 7, 8).

Nitsch and Nitsch (9) examined a variety of other possible test objects and compared their suitability with that of previously reported methods. These workers found that sections cut from first internodes of Brighton oats were especially sensitive to growth promoters, reliable in response, and applicable over a wide concentration range. Unfortunately, this variety is in very limited supply, and fresh seed generally is quite difficult to obtain.

The present report describes a very satisfactory first internode test which has proved to be valuable for detecting the growth stimulants obtained from plant extracts as well as in searches for new types of synthetic plant growth regulators.

MATERIALS & METHODS

BIOASSAY: Most of the oat varieties employed in these experiments were obtained from local feed dealers. The Brighton seed was harvested on the Union Carbide Research Farm near Clayton, N. C., from stock originally supplied by the Canadian Department of Agriculture.

Seed was embedded to a depth of 3 to 5 mm in quartz sand contained in 9 × 14 inch pyrex trays, and the sand was then saturated with distilled water with precaution to avoid free-standing surface pools. The planted trays were stored in total darkness at a controlled temperature of 70 to 72° F for 5 days. All subsequent manipulations were conducted either in total darkness or in green light (principally 546 mμ) under conditions, described previously (9), which are without notable effect on internode growth.

At the appropriate stage of growth, 4.0 mm sections of the first internodes were cut with a coleoptile microtome (9, 12) of Thimann's improved design. Following immersion in distilled water for about one hour, eight to ten sections were transferred to a 16 × 125 mm test tube containing the desired concentration of test compound in citrate-phosphate buffer at pH 5.0 to which 2 % by weight of sucrose had been added. The tubes were rotated at 1 rpm on a Wedco tissue culture roller for a period of time (generally 18 hr), the sections removed with a spatula, and their length measured with the aid of a photographic enlarger.

EXTRACTION OF GROWTH SUBSTANCES: Pea plants (*Pisum sativum* L., var. Alaska) were grown under the above conditions for 7 days after planting. The aerial parts were excised, washed with distilled water, immersed in purified methanol, and ground in a Waring blendor. The suspension was stored in the dark at 4° C for about two hours, filtered by suction through paper, and the filter cake washed with cold methanol and oven-dried to constant weight.

Organic solvent was removed from the filtrate under reduced pressure on a rotating evaporator at temperatures below 40° C, the aqueous residue was extracted three times with peroxide-free ether, and the combined ether layers were washed several times with distilled water and dried over anhydrous Na_2SO_4 in the refrigerator. This colorless solution was evaporated to a small volume in vacuo and an aliquot chromatographed and assayed in the same manner as the aqueous fraction.

The combined aqueous layers were evaporated to small volume under reduced pressure and an aliquot (generally 1 ml) placed as a single streak near the bottom of a dry sheet of Whatman no. 17 paper which had been washed thoroughly in dilute hydrochloric acid, distilled water, and acetone in that order. Chromatography was carried out in a commercial Chromatocab at 25° C, employing a mobile phase of isopropyl alcohol—conc. NH_4OH—H_2O (80:5:15 by vol) in the ascending direction.

After about 20 hours, the chromatograms were air dried, cut into 10 to 15 horizontal segments, and an equal part of each segment added directly to the tubes containing buffer and sucrose. Corresponding parts were treated with appropriate standard color reagents.

Further concentration of the active components of the aqueous fraction was accomplished by extracting the entire chromatogram with purified acetonitrile containing between 5 and 10 % methanol, followed by rechromatography on either Whatman no. 17 or no. 3 paper.

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Results & Discussion

Typical results obtained in growth experiments with first internode sections from several varieties of oats are compared in table I. The index was devised to express the relative value of each variety as a function of control growth, degree of elongation under chemical stimulation, and variability in both treated and untreated sections. A relatively high index indicates a large growth increment and a high degree of precision. From these data, it is apparent that the Forkedeer variety provides internode sections of outstanding quality.

This variety of red oat (*Avena byzantina* C. Koch) was developed at the University of Tennessee Agricultural Experiment Station by Stanton in 1930 and 1931 (6) as a winter-hardy selection. Unlike Brighton, it is commonly available throughout the Southern and Middle Atlantic states. In our experience, seedlings have been notable for exceptionally uniform growth rate and habit, high incidence of germination, complete absence of fungal infection, and insensitivity to minor temperature fluctuations. The husk does not appear to influence the sensitivity toward exogenous growth stimulants.

The effects observed through variation of several of the most important parameters affecting determination both of hormone profiles of plant extracts and the growth-promoting ability of new types of synthetic organic compounds are described in the following paragraphs.

Growth Rate: Figure 1 indicates the rate of growth of 4 mm Forkedeer sections under standard conditions, both in the presence and absence of added indole-3-acetic acid (IAA). Unlike Brighton sections, both treated and untreated objects cease to grow after about 16 hours, resulting in a constant difference between the two. This flexibility in the duration of treatment has been found very advantageous where a variation in daily laboratory routine becomes necessary.

Table I

Growth Characteristics of Oat Sections

Variety	Growth*: Control Length, mm	Control Std. Deviation	IAA (10^{-6} M) Length, mm	IAA (10^{-6} M) Std. Deviation	Elongation % of control	Index**
	A	B	C	D	E	F
Forkedeer	4.9	0.49	8.3	1.48	475	655
Clinton	4.6	0.44	6.3	1.73	410	540
Victory	4.5	0.48	6.4	2.44	520	445
Winter turf	4.5	0.48	7.7	3.98	695	365
Brighton	5.0	0.71	10.4	3.06	640	300
Tuxedo	5.2	0.87	9.3	1.79	450	290
Groats	5.0	1.05	8.3	2.91	440	140

* Initial section length 4.0 mm.

** $F = \frac{E}{BD}$

Effect of pH: The variation of sensitivity and growth with changing pH is shown in figure 2. Like Brighton, Forkedeer sections exhibit maximum response at a pH near four. However, the nearly parallel growth curves of the IAA-treated and untreated sections permit meaningful measurements of stimulation to be extended into the alkaline range. The significance of this observation may be demonstrated in investigations dealing with compounds such

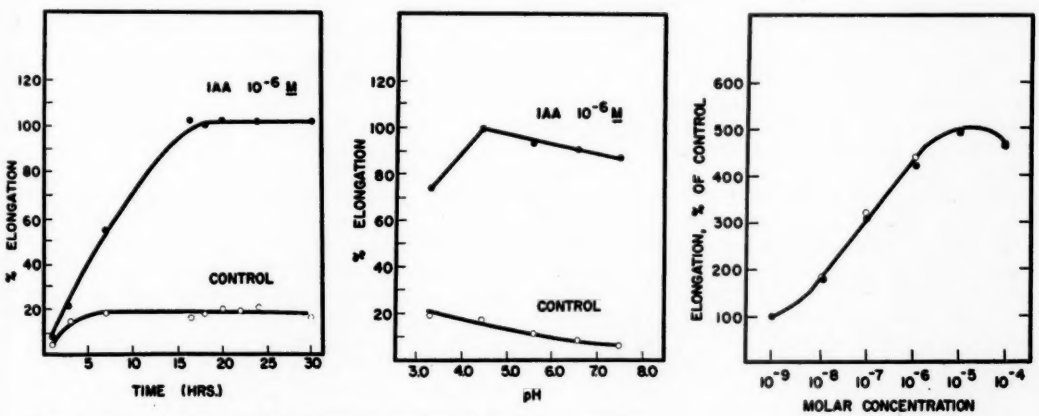

Fig. 1. Growth rate of Forkedeer oat sections in buffer + sucrose.
Fig. 2. Effect of pH on Forkedeer oat sections in buffer + sucrose.
Fig. 3. Effect of auxin concentration on oat sections in buffer + sucrose + 10^{-6} M IAA. ○ Forkedeer, ● Brighton.

as salts of organic acids and with hydroxy acids which form cyclic esters or lactones under acid conditions. Although most of our measurements have been made at pH 5.0, the shallow slopes of the pH-activity curves indicate that some latitude in buffering capacity is permissible.

Effect of Auxin Concentration: The ability of various concentrations of IAA to stimulate growth in sections of Brighton and Forkedeer oats is shown in figure 3. Although Forkedeer occasionally does not reach the maximum degree of stimulation observed in the other variety, the generally comparable response and range of sensitivity make it of exceptional value in the qualitative and semiquantitative evaluation of growth substance activity required in developing growth profiles of plant extracts by paper chromatography. Since, in most of these cases, the chemical nature of the growth promoters is not known with certainty, application of a truly quantitative measure becomes meaningless. In such instances, emphasis must be placed upon reliable detection of biological activity regardless of the chemical structure of the stimulant.

Effect of Synthetic Growth Regulators: Forkedeer sections have been found to respond to a variety of synthetic growth regulators over wide concentration ranges. In general, concentration-growth curves are similar to those of figure 3. Compounds which have been examined include the halogenated phenoxyacetic acids, substituted cinnamic acids (manuscript in preparation), the indole-3-alkanoic acids and amides (3), and many other substituted indoles.

Determination of Hormone Profiles: As an example of the utility of Forkedeer sections for determining the array of growth promoters extractable from plants, the hormone profiles of both ethereal and aqueous extracts of the pea (*Pisum sativum* L., var. Alaska) are shown in figure 4. The quantitative variation in response may be due to the intrinsic difference in stimulatory ability among active compounds, the presence of growth inhibitors exhibiting the same Rf as the promoters, variation in the concentration of promoters, or a combination of these factors.

Chromatography of the ethereal extract reveals a zone of activity which corresponds in Rf with IAA (Rf 0.44). While no color was observed upon application of Ehrlich's reagent to these chromatograms, the quantity of IAA present might be too small to detect. On the other hand, several careful investigations of pea extracts (5, 11) have failed to demonstrate the presence of IAA, and the stimulation observed in the present case may be due to another compound. It should be noted that the profile of the ether extract is similar to that found by Phillips and coworkers (10) who employed Brighton sections in their biological tests. However, an important factor overlooked by these investigators was the necessity for thorough removal of water from the ethereal layer prior to bioassay. We have found that even the small amount of water present in the organic layer (generally ca. 1.3 % by weight at 20° C) will permit solution of sufficient quantities of growth stimulants normally insoluble in ether to result in misleading interpretations.

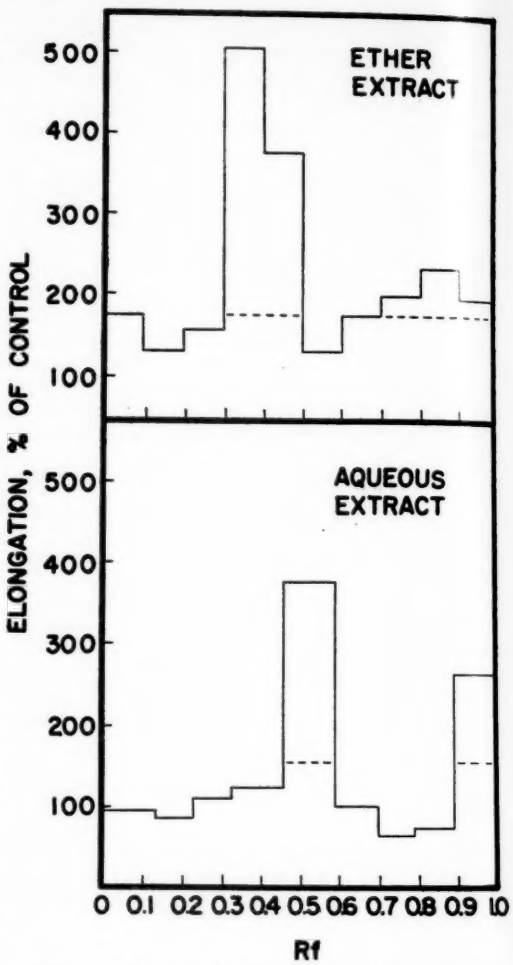

Fig. 4. Effect of Alaska pea extracts on growth of Forkedeer oat sections in buffer + sucrose. Values above the broken line are statistically significant at the 1 % level.

The aqueous residue remaining after extraction with ether provides several growth-promoting zones upon chromatographic examination. Although purple spots corresponding to several of these areas appear after treatment with Ehrlich's reagent (Rf. 0.34, 0.78, 0.89), Rf comparison with an extensive catalog of values for known indolic natural products still does

not provide firm evidence of their chemical nature.

Extraction of the thoroughly dried residue or its chromatogram with acetonitrile containing between 5 and 10% methanol, followed by paper chromatography, results in a hormone profile almost identical to that observed in the intact aqueous fraction. This leads us to believe that all of the growth-stimulatory substances of the Alaska pea may be accounted for in the organic extracts. The extended investigation resulting from these observations will be the subject of future communications.

Summary

I. Examination and comparison of the characteristics of first internode sections from a number of oat varieties has led to the development of a convenient bioassay for chemical growth stimulants, both synthetic and of natural occurrence.

II. The Forkedeer variety has been shown to provide sections of outstanding uniformity, sensitivity, range of effective concentration and pH, and availability.

III. Determination of hormone profiles of aqueous and ethereal extracts of the Alaska pea, based on the Forkedeer bioassay, failed to provide conclusive evidence of the presence of indole-3-acetic acid in this variety. Several other unidentified growth stimulants, insoluble in ether, were detected.

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EFFECT OF SODIUM BICARBONATE ON IRON ABSORPTION BY ORANGE SEEDLINGS[1]

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Previous studies have shown that some plants, when grown in nutrient solutions containing $NaHCO_3$, suffer reduced production of dry matter, reduced iron absorption, and chlorosis (1, 2, 3, 4, 5). All of these experiments involved one or more weeks of duration. Evidence is needed as to the initial influence of bicarbonates on absorption and translocation of iron.

Seedling plants of sweet orange, *Citrus sinensis* (Linn.) Osbeck, were grown in a glasshouse in 3-liter pyrex culture jars, two plants per jar and three replicate jars per treatment, using nutrient solution of the following composition (ppm): Ca, 50; Mg, 12; K, 20; N, 49; P, 4; S, 8; Fe, 1; Mn, Cu, Zn, B, 0.1 each. For the measurement of iron absorption the plants were transferred to similar culture vessels containing nutrient solutions that differed from the original in the following respects: A: Na_2SO_4 at the rate of 10 meq/liter was added to the control cultures, numbers one to three; $NaHCO_3$, also at the rate of 10 meq/liter, was added to the treatment cultures, numbers four to six; B: the iron was tagged with Fe^{59} (all as $FeSO_4$) at the rate of 0.21 mC/mg; C: phosphorus was omitted in order to minimize the tendency for iron to be precipitated.

The bicarbonate cultures received compressed air with 3.5 % of CO_2 added, which maintained the equilibrium with bicarbonate ion at pH 7.5. Control solutions received compressed air only and pH was held within ± 0.2 pH unit of this value by a single addition of normal HNO_3 during the course of the experiment. The pH was measured by glass electrode.

The possibility of precipitation of iron in the nutrient solutions was checked by making activity counts on samples after filtering through no. 42 Whatman paper. No differences were observed between the $NaHCO_3$ and Na_2SO_4 cultures and the only changes were accounted for by removal of iron and water by the plants. While this test does not preclude the possibility that part of the iron was in colloidal form, it does indicate that the iron remained distributed throughout the solutions for the duration of the experiment.

After 5 hours' exposure to the respective nutrient solutions (9 am–2 pm), the plants were harvested by cutting each stem at a point about one inch above

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TABLE I

IRON ABSORBED BY ORANGE SEEDLINGS DURING 5-HOUR PERIOD AS INFLUENCED BY $NaHCO_3$

JAR NO.	PLANT	LEAVES + STEMS		ROOTS	
		WT OF Fe* μg	CONC OF Fe* IN DRY MATTER ppm	WT OF Fe* μg	CONC OF Fe* IN DRY MATTER ppm
		Controls—Na_2SO_4 at 10 meq/liter			
1	A	15×10^{-3}	44×10^{-3}	8.5	42
	B	39×10^{-3}	87×10^{-3}	8.9	42
2	A	11×10^{-3}	27×10^{-3}	10.9	45
	B	13×10^{-3}	32×10^{-3}	11.2	49
3	A	3×10^{-3}	16×10^{-3}	5.7	36
	B	11×10^{-3}	21×10^{-3}	8.7	35
		Treated—$NaHCO_3$ at 10 meq/liter			
4	A	0	0	2.3	15
	B	0	0	5.3	20
5	A	0	0	2.6	13
	B	0	0	2.4	11
6	A	0	0	3.0	12
	B	0	0	4.2	18

the solution level. Roots were rinsed by dipping once in tap water; leaves and stems were washed with soap. After being dried (60° C) and weighed, the samples were ashed (500° C) and dissolved in 6 N HCl. Activity counts on these solutions were made in a well-type gamma scintillator and the results are given in table I.

The data show that substantial amounts of tagged iron moved into the tops of control plants but none was detectable in stems or leaves of plants from the bicarbonate cultures. Whether bicarbonate ion affects absorption of iron by roots or translocation from roots to tops is not clear. The fact that Fe* concentrations in roots of the bicarbonate-treated plants were lower than controls is at least consistent with the hypothesis that the effect was to reduce iron absorption by roots.

The results show that A: *Citrus sinensis* is among those plants that are sensitive to sodium bicarbonate, and B: the mechanism can be effective immediately upon exposure to the bicarbonate system.

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UPTAKE OF CALCIUM BY EXCISED BARLEY ROOTS [1, 2]

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The term non-metabolic uptake has been used to describe the uptake of ions not directly involving metabolic activity (10). There is a great deal of uncertainty involved in the measurement of non-metabolic uptake and likewise in the measurement of metabolic absorption. When a non-metabolic uptake correction is applied to large absorption rates, the resulting errors are not appreciable and can usually be ignored. However, when the correction is of the same order of magnitude as the total uptake, evaluation of the metabolic component may be exceedingly difficult.

Ca has long been recognized to be a slowly absorbed ion (8, 18). The reported total Ca uptake by excised barley roots (7, 9, 12, 19) is approximately equal to the non-metabolic uptake for other cations (10). Consequently, it is impossible to obtain a reliable measure of the metabolic absorption rate of Ca from total uptake data alone. For this reason, this study was designed to investigate the nature of the Ca uptake by excised barley roots with particular emphasis on the evaluation of the non-metabolic and metabolic components.

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[2] This paper is based on work performed in part under contract AT-(11-1)-34, project 5 with the Atomic Energy Commission.

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Materials & Methods

All these experiments were conducted with 6-day-old excised barley roots of the variety Tennessee Winter, 1956 crop. The method of culturing root material was similar to that described by Jacobson et al (14). The excised roots were washed several times in distilled water prior to the experiment. After washing, the roots were centrifuged at 65 × *g* for 5 minutes. Representative samples of roots were weighed out and placed in bottles of the desired salt solutions which were kept at constant temperature in a water bath. Unless otherwise stated, 7 grams of roots in 7 liters of solution were used. The pH of the solutions was rigorously controlled by adding acid or base throughout the experimental period. The resulting changes in solution composition were negligible. All experiments were done at 25° C., unless otherwise indicated. The solutions were aerated continuously during the experiment.

Stock solutions of $Ca(OH)_2$ were prepared by allowing saturated solutions of this base to stand in closed pyrex bottles for several weeks. Just prior to the experiment, portions of the clear supernatent were drawn off. Care was taken to avoid exposure of the $Ca(OH)_2$ to CO_2.

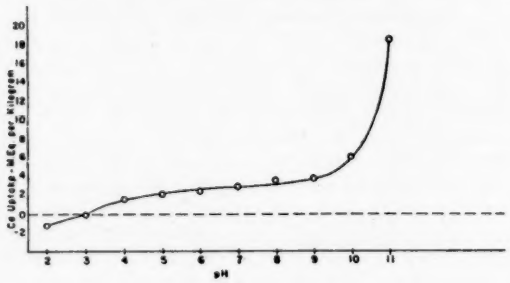
At the end of the absorption period, the roots were collected by pouring the contents of the bottles through a fine mesh nylon screen. The roots were washed in a running stream of distilled water for 10 seconds. Great care was taken to standardize the washing pro-

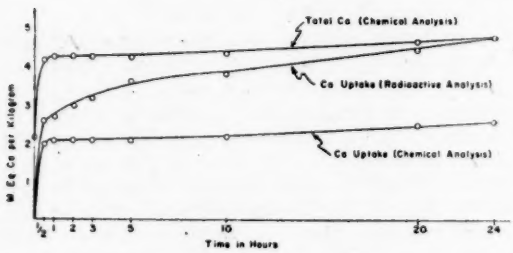
cedure to minimize variations in the non-metabolic uptake. The washed roots were dried in an oven overnight and ashed in a muffle at 600° C. K was determined by flame photometry on an acidified solution of the ash. Aliquots of the same unknown solutions were titrated with ethylenediamine tetraacetic acid to the Calcein endpoint for Ca (3).

Roots for organic acid analysis were frozen rapidly with liquid nitrogen at the end of the experiment. The material was lyophylized and organic acids were determined by ether extraction and titration (20). Inorganic ions were determined on ashed samples of the lyophylized material as described above.

When Ca^{45} was present in the samples, 1 ml of the unknown solution was placed in an aluminum planchet and dried in an oven. Duplicate planchets were prepared for each sample. Standards were prepared which had the same mass as the unknowns. Each planchet was counted twice in a windowless flow counter. Variations in count due to differences in mass and geometry were negligible. Counting rates were approximately ten times background.

Uptake data are expressed in meq per kilogram of fresh roots. These values represent the net change in content of the roots during the absorption period. Negative values indicate a loss of ions from the root. Initial contents of experimental material were 2.2 to 2.9 meq Ca and 11.2 to 13.4 meq K.





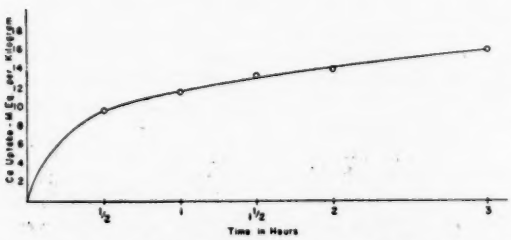


FIG. 1. The effect of pH on the uptake of calcium in 3 hours from 0.005 N $CaBr_2$

FIG. 2. The uptake of calcium as a function of time as measured by both chemical and radioactive analysis. The concentration of $CaBr_2$ was 0.005 N and the pH was 5.

FIG. 3. The uptake of calcium as a function of time from 0.005 N $CaBr_2$ at pH 11.

RESULTS & DISCUSSION

The effect of pH on the absorption of monovalent cations by barley roots has been studied in detail (11, 13). An experiment was designed to determine the effect of a wide range of pH on Ca uptake from a single salt solution. $CaBr_2$ and $Ca(OH)_2$ were combined so that the resulting solutions were 5 meq per liter in Ca at pH 11. These solutions were then adjusted to give a pH range from 2 to 11 by adding HBr. The uptake period was 3 hours. The results of this experiment are shown in figure 1.

It is apparent from figure 1 that there was little uptake of Ca below pH 9. Above pH 9 there was a rapid increase in Ca uptake. These data have not been corrected for non-metabolic uptake. The total uptake from pH 4 to 9 was of the same order of magnitude as the reported non-metabolic uptakes for other cations (10).

To obtain a measure of the non-metabolic uptake of Ca, experiments were conducted in which the metabolic absorption would be reduced to a low value. This was done by treatment with 2-4,dinitrophenol (DNP) and at low temperature. Since K is absorbed rapidly by this root material, the treatments were applied to K at the same time to measure the effectiveness of the treatments in inhibiting metabolic absorption. The uptake period was 3 hours and the concentration of Ca or K was 5 meq per liter. For the low temperature experiments the roots were submerged in distilled water at 0° C for 1 hour prior to the uptake period to eliminate any residual metabolic activity carried over from room temperature (15). The control and the DNP treatments were at 25° C. The pH was maintained at 5.0 throughout the experiment. The results are summarized in table I.

The results of table I indicate that most, if not all, of the Ca uptake by these roots was non-metabolic. It is difficult to conceive of a metabolic Ca absorption which is insensitive to both low temperature and DNP. If any metabolic component did exist, it was too small to be measured using this technique.

The conclusions drawn from the data in table I do not agree with those of Fried et al (6) and Chasson and Levitt (2). Chasson and Levitt found a rapid initial rise followed by a small linear increase with time up to 24 hours for the uptake of Ca^{45} by potato slices. Fried et al, using excised barley roots, have

TABLE I

EFFECT OF LOW TEMPERATURE AND DNP ON UPTAKE OF K & Ca

TREATMENT	TEST SOLUTION	CATION UPTAKE IN 3 HR AT pH 5, meq/kg
Control	KBr	23.5
0° C	KBr	3.0
10^{-6} M DNP	KBr	10.7
5×10^{-6} M DNP	KBr	0.9
10^{-5} M DNP	KBr	−0.4
Control	$CaBr_2$	2.0
0° C	$CaBr_2$	2.0
10^{-6} M DNP	$CaBr_2$	2.1
5×10^{-6} M DNP	$CaBr_2$	2.0
10^{-5} M DNP	$CaBr_2$	2.1

also reported a large initial uptake followed by a slow linear component for Ca^{45}. In both cases, the linear phase was interpreted to represent the steady-state metabolic absorption rate of Ca. The results of these investigators are open to serious objection, however. The uptake in both cases was measured using a radioisotope. If any appreciable isotopic exchange occurred with previously absorbed inert Ca, their conclusions would be in error. Such an exchange might be expected to occur, even over a longer period of time (16).

In an attempt to reconcile the differences between this study and the results of the above authors, an experiment was conducted using Ca^{45} to measure the uptake. In addition to counting the samples for Ca^{45}, total Ca was determined on the same samples by chemical analysis. Therefore, the results also give a measure of the isotopic exchange of Ca^{45} for inert Ca in the root. The uptake period varied from ½ hour to 24 hours and the pH was maintained at 5.0. The concentration of $CaBr_2$ was 5 meq per liter labeled with about 50,000 cps per meq. A parallel experiment was conducted at the same time in which the roots were given a 1-hour exposure to KBr after having been in the $CaBr_2$ solutions. The uptake of K was used as an indication of the metabolic activity of the roots. There was no reduction in the ability of the roots to absorb K, even after 24 hours in $CaBr_2$.

The total Ca content of the roots and the uptake of labeled Ca and inert Ca as a function of time are shown in figure 2. The striking feature of these curves is the large discrepancy between labeled Ca uptake and inert Ca uptake. Since there can be no doubt about the interpretation of the chemical analyses, these results point to a large isotopic exchange of Ca^{45} for initially present inert Ca. Furthermore, it is apparent that it took at least 24 hours for all the Ca in these roots to come to complete isotopic equilibrium.

The high initial uptake of Ca as measured by chemical analysis indicates a large non-metabolic component. Even though there is a small positive slope in the time curve after 1 hour, the rate of Ca increase is only about 0.1 meq per 5 hours. Two other long term experiments showed even slower rates. The small increase with time does not necessarily imply a metabolic process. It may be a reflection of exchange of Ca for Mg. Mg analyses indicated a slow loss of Mg over the entire time period. An alternative explanation is that growth may have occurred during the 24 hour uptake period. In addition to using Ca for new cell constituents, growth may have caused an increase in the total non-metabolic component. An increase in the non-metabolic uptake of K with time has been reported for similar barley root material (10).

Although the uptake of Ca at pH 5 by this tissue appears to be largely non-metabolic, an experiment was conducted to determine whether or not the isotopic exchange of Ca^{45} was a metabolic process. The experimental conditions were similar to those for figure 2. The time period was 3 hours and the concentration of DNP was 10^{-5} M. A KBr treatment was included to test the effectiveness of the DNP. The results showed that 10^{-5} M DNP completely inhibited the absorption of K. The effect of DNP on the isotopic exchange of Ca is shown in table II. These results show that the isotopic exchange of Ca was not affected by 10^{-5} M DNP. From this it would appear that Ca uptake and the isotopic exchange of Ca were both largely non-metabolic processes under the conditions of these experiments.

In light of the largely non-metabolic nature of the Ca uptake at pH 5, the uptake of Ca at high pH was investigated more thoroughly. Figure 3 shows the uptake of Ca as a function of time from a solution containing 5 meq $CaBr_2$ per liter at pH 11. Although the uptake curve is not linear, the continual increase with time suggested that the Ca uptake at this pH may have been an active process. At pH 11 the uptake of Br (about 1 meq/kg) can all be accounted for as non-metabolic uptake. Therefore, Br cannot serve to balance the uptake of Ca at this pH. Ordinarily, this root material balances excess cations absorbed by the production of organic acid anions (12) and this relationship may be used to measure metabolic absorption of a cation (10). The balancing of excess Ca uptake at pH 11 by organic acids would show that Ca was being metabolically absorbed at this pH. An experiment was designed to test this possibility.

TABLE II

EFFECT OF 10^{-5} M DNP ON ISOTOPIC EXCHANGE OF Ca

TREATMENT	3 HR IN 0.005 N $CaBr_2$ AT pH 5		
	TOTAL Ca BY CHEMICAL ANALYSIS meq/kg	Ca UPTAKE BY CHEMICAL ANALYSIS meq/kg	Ca UPTAKE BY Ca^{45} ANALYSIS meq/kg
Control	5.0	2.1	4.1
10^{-5} M DNP	5.0	2.1	4.1

Roots were treated in 5 meq $CaBr_2$ per liter at pH 11 for 3 hours. A parallel KBr treatment at pH 11 was included to give a measure of the ability of the roots to form organic acid anions at this high pH. The results of the KBr treatment indicated that the excess K absorbed at this pH was indeed balanced by organic acid anions. The Ca uptake from the $CaBr_2$ treatment was 17.2 meq per kilogram and the increase in organic acids was only 2.4 meq per kilogram. It appears, therefore, that excess Ca uptake at pH 11 did not elicit equivalent organic acid anion production, even though the tissue was capable of producing them. The loss of K and Mg from the roots during the $CaBr_2$ treatment was only 3.2 meq per kilogram and cannot account for the discrepancy.

The precipitation of $CaCO_3$ on or within the root was considered as a possible explanation of the large Ca uptake at pH 11. Although care had been taken to exclude external CO_2, the respiration of the roots may have supplied sufficient CO_2 to precipitate $CaCO_3$. To check on this possibility, 50 grams of roots were placed in 18 liters of 0.005 N $CaBr_2$ at pH 11. At the end of a 3-hour treatment, the roots were transferred to a closed vessel fitted with a KOH well. The system was evacuated and sufficient 1 N H_2SO_4 was introduced to cover the mass of roots. The system was kept closed and was occasionally agitated by swirling. At the end of 48 hours, the KOH was removed and titrated for the presence of carbonate. The results indicated that no more than 2 meq of Ca per kilogram of roots could be accounted for as $CaCO_3$. It is entirely possible that this figure is too large, since the strong acid treatment may have liberated CO_2 by decarboxylation of organic compounds.

A sample of roots which had been in a $CaBr_2$ solution at pH 11 for 3 hours was transferred to distilled water for 30 minutes. Out of a total Ca uptake of 18.4 meq per kilogram there was a loss of 12.3 meq. It would appear, therefore, that a large fraction of the Ca uptake at high pH is easily lost to distilled water.

Conclusions

Most, if not all, of the Ca uptake by this barley root material in the physiological pH range was non-metabolic. Ca has a marked influence on the absorption of other ions (5, 11, 19, 22). To be effective Ca must be present during the absorption process. Pretreatment of the roots with Ca is ineffective. This lack of a carry-over effect and the largely non-metabolic nature of the Ca uptake suggests that the action of Ca is in the surface region of the cell. As a result of this action, the permselectivity of the cell is greatly enhanced (11). The most logical place for this permselective barrier is at the plasmalemma. Such a barrier would afford the cytoplasm an excellent means of protection from the external medium. In addition, the properties of the permselective region could impart a large degree of specificity to the over-all ion absorption process. It is postulated here that this region is relatively impermeable to Ca.

Denying access of large amounts of Ca to the cytoplasm may benefit the cell. It has been reported that Ca inhibits many of the enzyme systems found in cells, particularly those which require Mg (4). Skeen (21) has shown Ca salts to coagulate the cytoplasm when they are introduced into the vacuole. On the other hand, Ca has been shown to be a requirement for certain enzymes (4). However, if this Ca were bound tightly by those enzymes in vivo, the amount of free Ca ions inside the cell might be kept low.

It has long been recognized that a continuous supply of Ca is essential for plant growth. In this respect, the utilization of Ca by growing tissue is undoubtedly closely associated with metabolic activity. However, in light of the results of this study, it is doubtful that Ca is actively absorbed by mature barley root cells and accumulated into the vacuoles. If the concentration of undissociated Ca in the cytoplasm or vacuole never exceeded the external Ca concentration, there would be no necessity for the direct expenditure of metabolic energy to explain the entry of Ca into the cell. It is conceivable that Ca ions, after slowly leaking past a relatively impermeable region into the cell, may become rapidly immobilized. The presence of Ca oxalate crystals in the vacuoles of some plant species (17) suggests this possibility.

The rather rapid equilibration of Ca^{45} with a substantial part of the inert Ca initially present in the roots indicates that much of the initial Ca may be located external to the cytoplasm. This hypothesis is also supported by the largely non-metabolic nature of this equilibration. A fraction of the initial Ca also appears to be readily exchangeable for other cations (10). Almost half the initial Ca in these roots may be in this exchangeable form, since that amount is easily lost to a solution containing 5 meq KBr per liter. If any of the remaining initial Ca is present as Ca pectates in the cell walls, then the amount of Ca inside the cytoplasm and vacuoles of these roots might easily be less than 1 meq per kilogram.

Except for perhaps that Ca which elicits the small increase in organic acids, Ca uptake at high pH was also largely non-metabolic. It is suggested that there are groups present in the barley root containing dissociable hydrogen which are capable of holding Ca at these high pH values. The Ca would be released by hydrolysis from these groups when the roots are placed in distilled water. Apparently, K is not held to any large extent by these groups since there was good agreement between K uptake and organic acid increase at pH 11. The nature of such groups and their significance, if any, to the mechanism of ion absorption is not clear.

The largely non-metabolic nature of Ca uptake by excised barley roots raises the question of how intact plants obtain their Ca. Some species of plants are known to take up large amounts of Ca. The grasses, however, are characterized by their low Ca requirement. It may be that the rate of metabolic absorption of Ca is sufficient to supply the needs of

the barley plant but is too small to be measured in relatively short-term experiments. It might be argued that the roots used in these experiments were already saturated with Ca and no further metabolic absorption was possible. This seems unlikely, however, in view of the low initial Ca status of these roots. Another possibility to account for the observed uptake of Ca by intact plants is surface migration through the root cortex to the transpiration stream. According to this hypothesis, an ion may move from the external solution along surfaces into the xylem without ever being actively absorbed. This may be more important for an ion like Ca than for other ions since much of the Ca appears to be associated with the cell surface region. There may be fixed negative charges in the cell wall as well as in the plasmalemma and Ca could migrate along these charged surfaces. Upon reaching the xylem, the Ca could be released by hydrolysis and carried upward in the transpirational stream. If these fixed charges are associated with phosphatides (1) and pectic substances, this type of movement could be rather specific for certain ions of which Ca may be the most important. Furthermore, the rate of surface migration of Ca through the cortex of the root need not parallel the rate of water movement through this region.

The complete equilibration of Ca^{45} observed in this study suggests use of caution when measuring absorption by means of an isotope. Furthermore, regardless of the location of Ca in the root or the mechanism by which it arrived there, it is clear that the uptake of Ca is a reversible process.

Summary

The uptake of Ca by 6-day-old excised barley roots appears to be largely non-metabolic. The Ca uptake at pH 5 was found to be insensitive to low temperature and dinitrophenol. The uptake of Ca^{45} was a reflection of isotopic exchange for initially present inert Ca in the root. This equilibration process was not affected by dinitrophenol.

There was a large uptake of Ca by this material at pH 11. This uptake was also largely non-metabolic. Only a small fraction of the Ca uptake at high pH could be accounted for by an increase in organic acids or by precipitation as $CaCO_3$.

It is postulated that much of the Ca in young barley roots is associated with the cell surface region. It is proposed that the Ca which is active in influencing the absorption of other ions is localized on this surface.

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INFLUENCE OF CALCIUM ON SELECTIVITY OF ION ABSORPTION PROCESS[1,2]

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INTRODUCTION

Of the various factors known to influence ion absorption by excised barley roots Ca is distinctive in that its effect depends upon the species of monovalent cation undergoing absorption (6). At a concentration of 5 meq/l, Ca is a very effective inhibitor of Li absorption and a moderately effective inhibitor of Na absorption. However, at the same concentration, Ca stimulates absorption of K, Rb, and Cs, particularly at low pH values. The latter phenomenon has been explained as primarily a blocking of a deleterious H uptake. It has been postulated that Ca modifies the permeability of cytoplasmic surfaces in such a way as to decrease the access of certain monovalent cations to the absorption site. According to this view the overall absorption process of monovalent cations, at least, initially involves a screening at the cell surface presumably non-metabolic in nature and affected by the presence of Ca followed by a metabolic absorption step (6). That the effect is due to a non-metabolic Ca fraction rather than absorbed Ca is suggested by the lack of carryover effect following pretreatment with Ca and by the observation that metabolic absorption of Ca occurs very slowly, if at all, with this material (9).

It has been known for some time that polyvalent cations other than Ca exert an effect similar to that of Ca on K absorption (4, 11). In the present study a comparison is made between the effect of several polyvalent cations and the effect of Ca on the absorption of K and Li.

The absorption of K and Na are affected differently by Ca in solutions of the separate ions (6). Wadleigh and Bower (12) were able to vary the K to Na ratio in intact plants by varying the Ca content of the solution. Epply (3) connected K and Na selectivity in Porphyra with Ca. Preliminary data in this laboratory indicated that in mixtures of K and Na the effect of Ca on the absorption of each ion was qualitatively similar to that obtained in single salt solutions; that is Ca increased the absorption of K and decreased the absorption of Na. Accordingly a more comprehensive study on the interaction of Ca with the K-Na system has now been undertaken.

A protective effect of traces of Ca on plant material has frequently been reported, e.g., Laties (8). The studies reported here include an examination of the effects of very small amounts of Ca on the absorption of various ions.

Finally a brief survey is conducted to see how widespread the effect of Ca is with respect to several plant species.

MATERIALS & METHODS

In most experiments excised barley (*Hordeum vulgare* L.) root material was used. Barley seed was soaked in aerated distilled water for 24 hours. The germinated seeds were spread on cheesecloth supported by a tinned and Amercoat (Amercoat Corp.) painted screen. The ends of the cheesecloth dipped into the solution. The seedlings were grown in the dark using a dilute nutrient medium consisting of 0.1 mmole/l each of KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$. The solution was gently aerated. Four days after transferring the seed to the cheesecloth, the nutrient solution was replaced with fresh solution. Two days later the roots were excised just below the seed, cut into approximately two centimeter lengths, thoroughly washed with distilled water, and centrifuged for 5 minutes at $65 \times g$. A ratio of 1 g root material to 1 liter of solution was employed. In most cases the pH of the treatment solution was adjusted to pH 6.0 and maintained at this pH by additions of the appropriate acid or base, e.g., HBr or KOH in a KBr solution. The concentrations of the test salts were not significantly changed by these additions. Unless otherwise stated the concentrations of the alkali metal salts were 5 meq/l and were present as the bromides. The absorption periods were 3 hours and the solutions were continuously aerated during this period. All experiments were performed at 25° C. At the conclusion of the treatment, the roots were poured on a fine mesh nylon screen and washed for 10 seconds with flowing distilled water. After drying and igniting, the ash was taken up in dilute HCl. The alkali metal cations were determined by means of a flame photometer. Direct titration of Ca or Mg plus Ca in solutions which had been in contact with root material gave erroneous results due to the presence of organic matter. In these cases solutions were first evaporated and digested with concentrated HNO_3 and 30 % H_2O_2. Ca was then determined by titration with sodium ethylenediamine tetraacetate using Calcein indicator (2). Mg plus Ca was determined by Na EDTA titration with Eriochrome Black T as the indicator (1). Mg content was obtained by difference between this value and the Ca content.

In the data reported here no corrections for non-metabolic uptake have been applied. Since the pres-

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[2] This paper is based on work performed under Contract No. AT-(11-1)-34, project 5 with the United States Atomic Energy Commission.

ence of polyvalent cations may modify the nonmetabolic uptake of the accompanying monovalent cations, it was thought best not to attempt a correction. In any event the correction, based on previous experience with barley root material, would not be greater than 0.9 meq/Kg fresh weight (7). In most cases the correction would not be significant and in no case would the application of this correction alter the conclusions drawn from the data.

Results

In the first experiment the effect of various polyvalent cations on the absorption of Li and K was examined. These experiments were performed in single salt solutions of LiBr and KBr. The concentrations of the salts were 10 and 5 meq/l, respectively. Polyvalent cations were added as the bromide salts at a concentration of 1 meq/l except Mg at 2 meq/l. For most of the tests the pH was maintained at 6.5, but because of the limited solubility of Mn and Al ions at this pH, these two ions were tested at pH 4.7. At both pH values measurements of Li and K absorptions in the absence of any added polyvalent cation were included for purposes of comparison.

The uptake data, expressed as meq/Kg fresh weight, are given in table I. With the exception of Mg all polyvalent cations tested reduced the absorption of Li sharply. Mg, at the concentration used, had a relatively minor inhibitory effect on Li absorption. All polyvalent cations examined caused an appreciable increase in K absorption. In contrast to previous experiments (6) the variety of barley used in this experiment (Arrivat 1958 crop) showed an increase in K uptake resulting from the addition of Ca at pH 6.5.

Table I

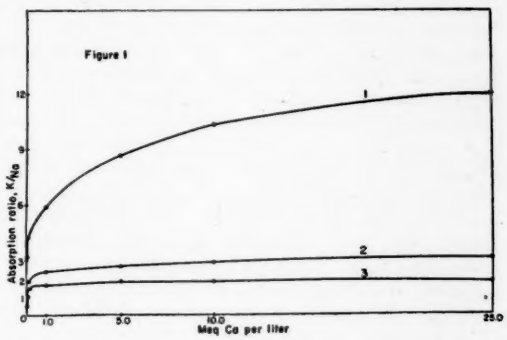
Effect of Various Polyvalent Cations on Absorption of Li & K by Barley Roots

Polyvalent cation	Absorption meq/Kg fresh wt*	
	Li**	K***
None	14.7	24.3
Mg	13.0	29.4
Ca	4.6	27.2
Sr	4.9	27.2
La	7.1	30.1
None†	12.3	22.7
Mn†	5.3	26.6
Al†	6.9	25.8

* Three hour absorption period.
** From LiBr at 10 meq/l + 1 meq/l polyvalent cation except Mg at 2 meq/l.
*** From KBr at 5 meq/l + 1 meq/l polyvalent cation except Mg at 2 meq/l.
† pH = 4.7, all others at pH 6.5.

Since earlier experiments suggested that Ca might control the relative amounts of Na and K absorbed from a mixture of the two, an experiment was undertaken to explore this behavior more fully. Barley root material (Tennessee Winter 1958 crop) was exposed to three series of solutions. All solutions contained 5 meq/l KBr. In one series 1 meq/l of NaBr was present. The second and third series contained 5 and 15 meq/l NaBr. In each series the Ca was varied from no Ca added to 25 meq/l. The pH was maintained at 6.0.

The data for the three series are presented in figure 1. In order to clearly show the effect of Ca on the relative absorption of Na and K, the ratios of absorptions of the two ions are plotted against the Ca added to the solution. It is immediately apparent that Ca exerts a large degree of control over the relative



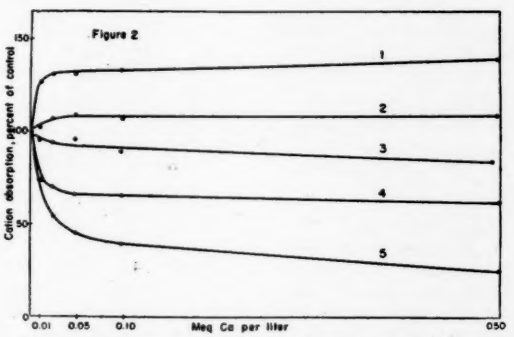


Fig. 1. Effect of Ca on the ratio of absorption of K to Na by barley roots. All solutions contained 5 meq KBr/l. In addition, treatments 1, 2, and 3 contained 1, 5, and 15 meq NaBr/l, respectively. The absorption period was 3 hours and the pH was maintained at 6.0.

Fig. 2. Effect of small amounts of Ca on the absorption of Li, Na, and K by barley roots. 1. K from NaBr + KBr. 2. K from KBr. 3. Na from NaBr. 4. Na from NaBr + KBr. 5. Li from LiBr. All alkali metal salts were present at 5 meq/l. The control for each curve is the absorption in the absence of added Ca. The absorption period was three hours and the pH was maintained at 6.0.

absorption of the two ions. It is also evident that the major effect occurred at the lower concentrations of added Ca. The total absorption values, i.e., Na+K, in the absence of added Ca were 30.1, 35.7, and 41.8 meq/Kg fresh weight from the K solutions containing 1, 5, and 15 meq/l Na, respectively. The corresponding total absorption values for Na+K from solutions containing 25 meq/l Ca were 40.2, 40.0, and 43.4 meq/Kg.

A study was made of the effect of Ca concentrations below 0.5 meq/l. The effect of Ca concentration on the absorption of Na and K from single salt solutions at 5 meq/l and from mixtures of the two at 5 meq/l each were examined. In addition to these ions, the effect of very low concentrations of Ca on the absorption of Li was studied. In these experiments Tennessee Winter 1958 crop barley was used.

The results are presented in figure 2. Because of the small amounts of Ca added, the Ca liberated by the root material into the test solution was a significant contribution to the Ca concentration of the solution. For this reason samples of the test solution were taken at the end of the absorption period and analyzed for Ca as described above. No allowance was made for the Mg released by the roots since the amount was too small to have an appreciable effect. Even without added Ca the solutions were not completely Ca free due to Ca release from the roots and the presence of Ca impurity in the salts. With no added Ca, the Ca concentrations at the end of the absorption period were 0.0011, 0.0015, 0.003, and 0.0019 meq/l for the NaBr, KBr, NaBr + KBr, and the LiBr solutions, respectively. The absorption curves of figure 2 have been plotted as percent of control.

TABLE II

EFFECT OF Ca ON ABSORPTION BY DIFFERENT PLANT SPECIES

	ABSORPTION, meq/Kg FRESH WT*					
	Na**		K**		Li***	
	−Ca	+Ca†	−Ca	+Ca†	−Ca	+Ca†
Barley, *Hordeum vulgare*	12.4	6.4	16.5	24.5	9.9	1.3
Corn, *Zea mays*	12.9	8.7	1.8	10.6	5.9	1.3
Onion, *Allium cepa*	11.4	3.3	−3.7	−0.7	9.8	1.0
Pea, *Pisum sativum*	8.0	4.1	−0.2	1.8	7.2	1.5
Squash, *Cucurbita maxima*	21.8	5.0	8.0	34.8	17.1	1.5
Sunflower, *Helianthus annuus*	18.8	8.4	−5.2	1.5	21.6	1.5

* Three hour absorption period.
** From mixture of 5 meq/l each of NaBr and KBr.
*** From 5 meq/l LiBr.
† Ca added at 5 meq/l as $CaBr_2$.

In each case the control is the absorption with no added Ca. The curves bring out the fact that Ca has a considerably greater effect on Na and K absorptions from a mixture of the two ions than from single salt solutions of the same ions. Comparing curves 1 and 2, with Ca at the 0.025 meq/l level, K absorption is 130 % of the control when absorption occurs from a mixture of Na and K. From KBr solution the absorption is only 106 % of the control. At the same Ca level, curves 3 and 4, Na absorption is reduced to 70 % of the control in the mixture and to 93 % in NaBr solution. The absorption values for the controls were 20.1 meq Na/Kg from NaBr, 23.0 meq K/Kg from KBr, 12.4 meq Na/Kg, and 16.5 meq K/Kg from the mixture. The total absorption, i.e., Na + K, from the mixture varied from 28.9 meq/Kg with no added Ca to 30.7 meq/Kg with 0.5 meq/l Ca. Curve 5 in figure 2 shows the absorption of Li from LiBr as a function of Ca concentration. The control for this curve was 10.1 meq Li/Kg. The high degree of sensitivity of Li absorption to minute concentrations of Ca is well demonstrated by this curve.

The above experiments were performed using barley as a test material. In order to see if the Ca effect was specific for barley or was a more general phenomenon, the effect of Ca on absorption was determined for six different species of plants. The results are listed in table II. The roots were from 6 day old plants grown and prepared as described for barley. The solutions consisted of LiBr and a mixture of NaBr and KBr: each without added Ca and with 5 meq/l Ca. To a greater or lesser degree all species responded to the addition of Ca. The response of squash roots to Ca is especially noteworthy. Additional tests using a level of 0.05 meq/l Ca likewise indicated a response to Ca. Attempts to use potato disks were unsuccessful because of the very large ratio of free space uptake to active absorption.

DISCUSSION

Other polyvalent cations share with Ca the property of altering the selectivity of barley root tissue to the absorption of monovalent cations. Of the polyvalent cations tested Mg was the only exception. It has been postulated that Ca modifies the selectivity of the cell membrane for monovalent cations (6, 9) presumably by reacting with the membrane. Since most of the polyvalent cations have a similar effect on selectivity, either the reaction is non-specific or these ions exert an effect via a Ca interaction. It is possible that polyvalent cations exchange for Ca in the root. If the polyvalent cation satisfies exchange sites which normally compete for Ca with the active binding sites that control selectivity, then even the small amounts of Ca liberated from inactive sites might be highly effective.

In a previous article (6) it was shown that the presence of Ca affected the absorption of Na and K from single salt solutions in quite different ways. Absorption of the former was reduced and that of the latter increased by the addition of Ca. The data reported here show that the same holds true for absorp-

tion from mixtures of Na and K. The ratio of absorption of the two ions is strongly influenced by the presence of Ca even if present in low concentration.

The absorption system is exceedingly sensitive to very small concentrations of Ca in the external solution. The addition of as little as 0.001 meq/l of Ca to solutions of LiBr and NaBr + KBr consistently reduces absorption of Li or Na about 3 %. Perhaps if it were possible to work with truly Ca free systems even greater sensitivity might be attained. It is interesting that Ca at a concentration of about 10^{-6} M is as good or better an inhibitor of Li and Na absorption than many metabolic inhibitors.

In a mixture of Na and K salts, the effect of Ca is considerably amplified compared to its effect in single salt solutions. A reduced access of Na and an increased access of K caused by the presence of Ca would result in presenting to the absorption site an increased ratio of K to Na. It is this increased ratio which may cause the enhancement of the Ca effect in Na and K mixtures. Inherent in this view is the supposition that Na and K compete for the same metabolic ion carrier. It is suggestive that in the presence of varying amounts of Ca the sum of the absorptions of Na and K remained constant within about 6 % although the ratio of absorption of the two ions varied from 1.33 to 3.00. This is the type of behavior one would expect if a common carrier were involved.

The particular Ca reaction which controls the relative selectivity has not been ascertained. There is no scarcity of substances in cellular membranes which are capable of reacting with Ca (10). The problem appears to be which substances and what mechanisms are involved. It has been suggested that RNA in cell membranes is involved in ion accumulation and that divalent ions protect the RNA from degradation by endogenous enzymes (5). It is possible that membrane RNA is involved in the Ca effect, but the inhibitory effect on Na and Li absorption appears to preclude the role of Ca as a simple protective agent.

It is probable that the type of absorption response to Ca described here is widespread among plant species. All root material thus far tested behaves in an essentially similar fashion. It is reasonable to assume that Ca has a great deal to do with the preference that many plants exhibit for K over Na.

Summary

Several polyvalent cations affect absorption similarly to Ca. Mg appears to be an exception. It is suggested that either the particular mechanism involved is not specific for Ca or that the polyvalent cations act via a Ca interaction.

Ca was found to have the property of drastically altering the ratio of absorption of Na and K from a mixture of the two. Using Li and Na + K solutions it was shown that the absorption system was extremely sensitive to small concentrations of Ca. As little as 10^{-6} M Ca was sufficient to cause a detectable change in absorption rates. The effect of Ca is enhanced in mixtures of Na and K compared to its effect in single salt solutions.

The essentially constant sum of the absorptions of Na and K in spite of large changes in the ratio of absorbed Na and K caused by the presence of Ca suggests a common metabolic carrier.

The controlling behavior of Ca was found in the roots of six different species of plants. This may indicate a widespread occurrence.

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EFFECT OF ROOT TO SOLUTION RATIO IN ION ABSORPTION EXPERIMENTS[1,2]

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Relatively little attention has been paid in ion absorption studies to the ratio of absorbing tissue to solution volume. In most cases it appears to be largely determined from the standpoint of convenience. In studies with root material ratios varying from 1 to 100 g per liter solution have been used (1, 5, 7, 8). It is possible that differing results and conclusions based on these results reported from various laboratories may be related to this factor. The likelihood that root to solution ratios might be critical is strengthened by the observation that very small concentrations of Ca have pronounced effects on absorption and root tissue tends to liberate Ca into the ambient solution (3). In the following experiments various factors which effect absorption and may be related to root to solution ratios have been examined. These include aeration, pH effects, and the liberation of active substances from the roots.

MATERIALS & METHODS

Excised barley roots (*Hordeum vulgare* L. var. Tennessee Winter) were used throughout this study. The material was grown and prepared as previously described (3). In all cases bromide salts were used and the concentration of the alkali metal salts was 5 meq/l. Except in those experiments dealing specifically with aeration, the solutions were vigorously aerated during the absorption period. A constant pH of 6.0 was maintained by additions of the appropriate acid or base, e.g., HBr or LiOH in a LiBr solution. However, in one experiment the pH was deliberately allowed to change as a result of root activity. Each absorption treatment lasted 3 hours and was performed at 25° C.

Most experiments were carried out in pyrex bottles or beakers. The root to solution ratios varied from 1 to 50 g per liter. In no case were less than 3.2 g root material or 1 liter solution used. Flow treatments consisted of 5 g roots in 100 ml capacity vessels through which the solution flowed. In these experiments the flow rates were 500 ml/min for the first 15 minutes, 200 ml/min for the next 15 minutes, and 50 ml/min for the remaining 2.5 hours. The flowing solution in contact with the roots was vigorously aerated. The pH of the stock solution was adjusted so the pH in the absorbing vessel averaged about 6.0. The influent and effluent solutions differed by 0.2 to 0.4 pH units.

At the conclusion of the absorption period, roots were caught on a nylon mesh screen, washed for 10 seconds in running distilled water, dried, and ignited. The ash was taken up in dilute HCl. The alkali metal cations, Ca, and Mg were determined as previously described (3).

No corrections were made for apparent free space or non-metabolic uptake. The amount of such uptake is small in this material, about 0.9 meq/Kg fresh weight. This value would not affect the conclusions.

RESULTS

In the first experiments adequacy of aeration was checked as a function of root to solution ratio. It was found that strong bubbling with either air or a 50 % O_2-N_2 mixture in root to solution ratios of 50 g/l gave identical absorption values for K. Slow bubbling with air resulted in lowered absorption rates at root to solution ratios above 10 g/l. It was concluded that even at 50 g/l aeration was not a limiting factor provided it was maintained at a vigorous rate.

The absorption of Na and K from mixtures of the bromide salts and Li from LiBr was determined at different root to solution ratios (fig 1). The very considerable effect of the root to solution ratio is apparent. There is a marked increase in K absorption and an approximately corresponding decrease in Na absorption as the root to solution ratio increases. With 1 g of roots per liter of solution the ratio of K

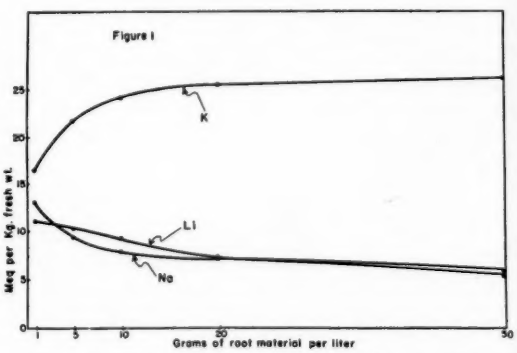

FIG. 1. Effect of increasing root to solution ration on the absorption of Na and K from a mixture of NaBr and KBr, each at 5 meq/l and of Li from 5 meq/l LiBr. Absorption period was 3 hours and the pH was maintained at 6.0.

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[2] This paper is based on work performed under Contract No. AT-(11-1)-34, project 5, with the United States Atomic Energy Commission.

to Na absorbed is 1.26; with 50 g/l it is 4.48. Li absorption with 50 g/l is decreased to less than half compared with absorption at 1 g/l.

A similarity exists between these curves and those obtained by varying the Ca content of solutions containing 1 g/l roots (3). Analysis of the ambient solutions used in the experiment from which the data presented in figure 1 were obtained gave Ca concentrations increasing from 0.001 to 0.03 meq/l as the root to solution ratio increased. Since Ca in this concentration range has pronounced effects on absorption, attempts were made to relate the absorption at different root to solution ratios to the amounts of Ca liberated from the root. The absorption rates occurring at various root to solution ratios were compared on the basis of final ambient Ca concentration with the rates in solutions containing 1 g/l of root material and the appropriate Ca concentration. This was considered valid since most of the Ca was liberated at an early stage of the absorption period. Good agreement was obtained for Li, fair for Na, and rather poor for K.

TABLE I

ABSORPTION FROM ROOT TREATED SOLUTIONS

CATION ABSORBED	meq/Kg FRESH WT*		
	ROOT TREATED SOLUTION**	SIMULATED SOLUTION**	MINUS Ca & Mg SOLUTION**
Li	8.0	7.5	9.2
Na***	11.1	10.9	14.8
K***	21.7	16.3	14.9

* Three hour absorption period.
** See text for description.
*** From mixture of NaBr and KBr.

In another experiment roots at a 20 g/l ratio were allowed to absorb from solutions of LiBr and KBr + NaBr for 3 hours. At the end of the absorption period the roots were removed from the solution and an aliquot of the latter analyzed for Ca, Mg, Li, or Na and K. In table I these are designated as root treated solutions. Simulated solutions were made up having the same composition of cations added as the bromide salts. Absorption experiments using 1 g/l of roots were performed using the root treated solution, the simulated solution, and a comparison solution of the same alkali metal salt concentration containing no added Ca or Mg. The results of this experiment are presented in table I. The data indicate that in addition to Ca and Mg other substances which modify absorption may be liberated from the roots. K absorption is the most affected. Recent work in this laboratory indicates that PO_4 at low concentrations, i.e., 5×10^{-6} M, stimulates absorption especially that of K.

In order to eliminate as much as possible the effect on absorption by substances originating from the roots, a flow experiment was carried out. The apparatus contained 5 g roots. Solution was flowed through at the rates earlier described. At the same time absorption was determined at root to solution ratios of 50 g/l and 1 g/l using fixed volumes. A comparison of the absorption values in table II verifies the conclusion that roots release substances capable of affecting absorption. The fixed volume treatment at 1 g/l resulted in lower Na and higher K absorptions than in the flow treatment. This suggests that even with 1 g of roots per liter, Ca and possibly other substances are liberated into the solution in sufficient quantity to have some effect on absorption.

TABLE II

EFFECT OF FLOWING SOLUTIONS ON ABSORPTION

CATION ABSORBED	meq/Kg FRESH WT*		
	FLOW	50 g/l FIXED VOLUME	1 g/l FIXED VOLUME
Li	10.1	5.17	10.1
Na**	13.4	5.80	11.9
K**	14.6	26.0	17.7

* Three hour absorption period.
** From mixture of NaBr and KBr.

Excised barley roots almost always absorb larger amounts of cations than anions from salts of monovalent cations. The pH of the solution will drop as a result of the excess cation absorption. A curve showing the decrease in pH of a KBr solution containing 20 g/l roots is given in figure 2. The initial pH was 6.0; the pH at the end of 3 hours was 4.3. The absorption of K amounted to 20.9 meq/Kg. With the same root to solution ratios but with the pH maintained at 6.0, 25.9 meq K/Kg was absorbed. At 50 g/l of roots the pH dropped from 6.0 to 3.7 in 3 hours.

Throughout these experiments it was observed that the amount of Ca liberated from the roots varied depending upon the composition and concentration of the solution. Table III contains data relative to this

TABLE III

EFFECT OF CONCENTRATION & SPECIES OF CATION ON Ca LIBERATION FROM ROOTS

TREATMENT	CONC OF SALT meq/l	CONC OF Ca LIBERATED meq/l*
LiBr	5.0	0.0018
NaBr	5.0	0.0011
KBr	5.0	0.0015
NaBr + KBr	5.0 each	0.0030
LiBr**	1.0	0.0080
LiBr**	10.0	0.014

* Three hour treatment period.
** 20 g roots/l, all others 1 g/l.

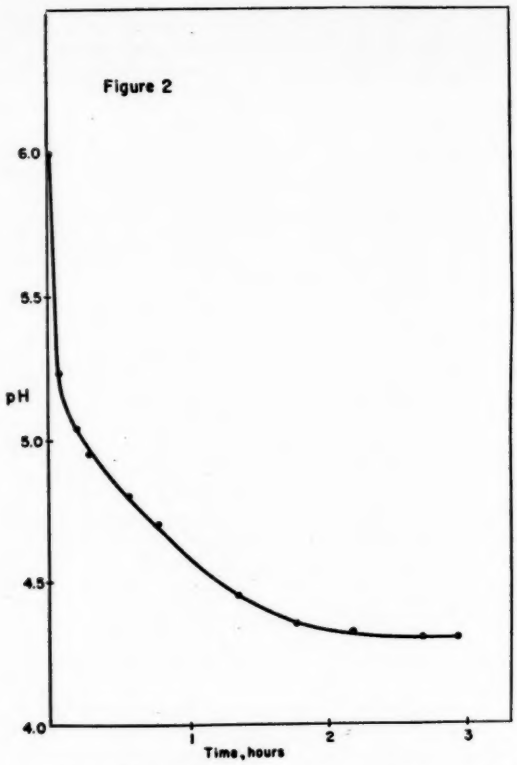

FIG. 2. Decrease in pH as a result of absorption by 20 g roots from 1 liter of solution containing 5 meq KBr.

point. For a given root to solution ratio higher salt concentrations result in increased Ca release from the root. The resulting differences in Ca concentration are sufficiently large in some cases so they can be expected to have a decided effect on absorption (3).

DISCUSSION

The findings reported here indicate that the root to solution ratio employed in absorption experiments plays a major role in determining the absorption behavior. With vigorous aeration gaseous exchange with the solution is sufficient to meet the respiratory needs of even high root to solution ratios. The changes in salt concentration as a result of absorption are too small to produce the observed behavior and are often in the wrong direction. The only other possibilities are the loss of active material from the roots and the transfer of absorption modifying substances from an inactive to an active site. The amount of these substances in the ambient solution appears to be a measure of this. H, Ca, and probably PO_4 can account for most of the observed effect.

The pH of unbuffered solutions may change drastically as a result of unequal absorptions of cations and anions and this change may affect the absorption rate. With barley root material in monovalent cation salt solutions, the pH almost always decreases because of more rapid cation absorption. In the absence of pH control the pH should drop until cation and anion absorption rates become equal since in this material cation absorption falls off more rapidly with decreasing pH than anion absorption (5). At high root to solution ratios the situation is even more complex because of the concomitant release of Ca from the roots. Ca would tend to decrease the effect of low pH for some cations and increase it for others (4).

The high degree of sensitivity displayed by absorbing roots to small amounts of Ca coupled with the observation that Ca release occurs from roots in salt solutions suggests that this element may be the major modifying factor. That this is so seems clear in the case of Li and Na absorptions. The Ca concentration of the ambient solution increases with increasing root to solution ratio. The concurrent decrease in absorption is quite close to what one would expect based on the Ca content of the solution. Other substances liberated from the roots in addition to Ca appear to affect the absorption of K. Recent work here indicates that trace amounts of PO_4 may be involved.

The fact that absorption rates may be subject to several uncontrolled factors due to experimental procedure creates difficulties in the interpretation of quantitative relationships. A case in point is the competition between Na and K. At minimal root to solution ratios, i.e., flowing solutions, Na and K absorption rates from an equimolar mixture of the two ions are about equal. With the same solution at the same pH, temperature, and time but with a fixed volume of 1 liter per 50 g roots about 4.5 times as much K as Na is absorbed. Obviously, very different conclusions would be drawn depending upon the root to solution ratio used.

The composition of the media has a determining influence on the amount of Ca and other absorption active substances originating from the roots. This is particularly important at the higher root to solution ratios where the amount of Ca involved is sufficiently large to exert a strong effect on the absorption rates. The amounts of Ca are also related to such factors as the ion species present, salt concentration, and undoubtedly the initial Ca status of the tissue. Not only does this salt effect have important consequences in connection with competition studies but also challenges the validity of certain types of kinetic studies. Attempts have been made to apply kinetic analyses employing the Michaelis-Menten and similar formulations to the ion absorption process in roots (1, 2, 6). The formulation presupposes the salt concentration as the only variant. It appears that this condition has not been met for the reasons stated above. Much of the absorption behavior which has been explained in terms of enzyme-substrate analogies and multiple site absorption is more likely due to the combination of high root to solution ratios and the salt effect on Ca liberation. Using low root to solution ratios and controlled pH, we have been unable to obtain the

linear reciprocal plots described by others (1, 6) as characteristic of absorption by roots. The foregoing discussion applies to alkali metal cations. We have not examined the situation for other ions.

The observations described in this investigation raise some serious problems insofar as certain aspects of ion absorption are concerned. These problems may be partially but not completely alleviated by using low root to solution ratios and controlling pH values. In general, it seems that the quantitative interpretation of absorption data must take into account the effect of factors arising from experimental design and some of these factors may not be readily amenable to control.

Summary

Large differences in absorption behavior are caused by changing the root to solution ratio in absorption experiments. Aeration ordinarily is not a factor. Changes in pH as a result of absorption activity cause a reduction in absorption rates. This factor may be eliminated by appropriate control measures.

The liberation of Ca and other absorption modifying substances from the roots is the most important factor. Much of the effect of increased root to solution ratios on absorption can be accounted for by Ca liberation. The composition and concentration of the absorbing solution are important in determining the amount of Ca released from the roots.

The fact that ion absorption is subject to such influences raises the question of the validity of certain types of kinetic studies and in general complicates the interpretation of all quantitative absorption data.

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EFFECT OF VARIOUS CATIONS UPON ABSORPTION OF CARRIER-FREE CESIUM[1, 2]

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INTRODUCTION

Because of the fallout problem a number of studies have been made of the behavior of the fission product, Cs^{137}, in soil and plant systems. And while there is no doubt that research along these lines is highly essential from the point of view of radiological safety, it is also true that this isotope is an important tool for more basic research. This is the case because cesium is an alkali element resembling the essential plant nutrient, potassium, and because of the experimental conveniences arising from the fact that the native Cs content of soils and plants is extremely low.

The recent work of Bange and Overstreet (1) has uncovered certain fundamental relationships in cesium absorption and its interaction with the absorption of other cations. Their experiments, however, dealt entirely with macro amounts of Cs.

The researches described below dealing with carrier-free Cs^{137} were conducted largely for two reasons. First, the isotopes of cesium in fallout are essentially carrier-free and thus such experiments have perhaps more practical significance. Second, there is considerable scientific interest attached to the fact that trace amounts of an element often behave differently in biological systems from macro amounts. In many respects the following experiments were patterned after those of Bange and Overstreet so as to reveal any differences of this nature.

MATERIALS & METHODS

The excised roots of Tennessee Winter barley, 1956 crop, prepared as described by Jacobson et al (2) were used in these experiments. One gram of root material in 1.0 liter of solution was used. To avoid the adsorption of Cs^{137} on glass, polyethylene containers and aerators were used. During each experiment the solutions were aerated constantly and maintained at 26° C ± 0.1° C by a water bath. To prevent marked depletion of the solutions with respect to carrier-free Cs^{137}, the competitive experiments were limited to 1 hour. The data (fig 1) indicate that absorption is essentially linear with time over this period even though as much as 30 % of the Cs^{137} is absorbed from the medium. The initial pH was approximately 5.5. Moreover, because of the ratio of root material to solution employed and because of the short length of time involved, the pH did not change appreciably during the experiments.

After immersion in the solutions for 1 hour, the roots were removed by a nylon mesh filter, washed briefly with distilled water (about 10 sec) at room temperature, and set aside on filter paper. After standing for about two hours, the roots were limp and easily forced into planchets for counting by means of a plexiglass funnel which was constructed to seat firmly over the planchets. The partially dried root material was gently forced into the planchets by means of a plexiglass pestel of the same diameter as the planchets. After the root material had been so inserted, it was dried at 70° C overnight. After drying, each planchet was covered with a mylar film. This was accomplished by placing a circle of mylar of diameter slightly greater than that of the planchet over the roots and forcing a ring of spun nylon over it and the planchet. The nylon rings were of such dimensions as to fit snugly around the rim of the planchet and hold the mylar film securely in place. The film served two purposes: it prevented spilling of roots inside the counting chamber and also it pressed down the dried root mass so that its surface was in every case flat and even with respect to the top of the planchet, thus preventing errors due to differences in geometry. The Cs^{137} gamma emission was measured using a Berkeley Decimal Scaler (Model 1001) and lead-shielded scintillation detector.

Except in the case of Rb, the salts were of analytical reagent grade. Rubidium chloride was obtained from Fisher Scientific Co. in the grade listed by them as "purified". The water used in preparing solutions was laboratory distilled, further purified by passage over a Dowex-50 resin column to remove traces of copper. All ions investigated were presented as chlorides.

Cs^{137} was obtained from Oak Ridge National Laboratory as CsCl in HCl. It was used at a concentration of 1.00 μc per liter.

EXPERIMENTAL RESULTS & DISCUSSION

The absorption of carrier-free Cs as a function of time in the absence of competing ions is shown in figure 1. The rate of absorption is seen to be constant over a 1 hour period and to have declined only slightly at the end of 2 hours in the solution, although by this time the solution has been depleted to approximately 50 % of the original activity. The absorption of Cs^{137} at 2° C is also shown in figure 1. Only about

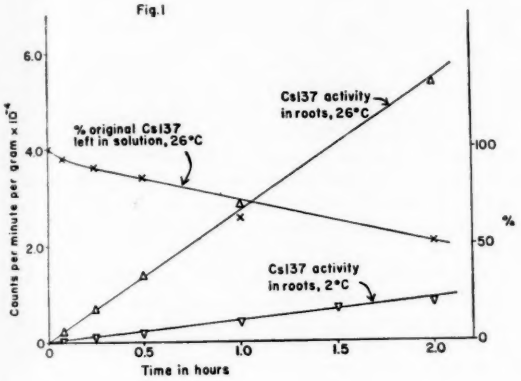
[1] Received August 23, 1960.

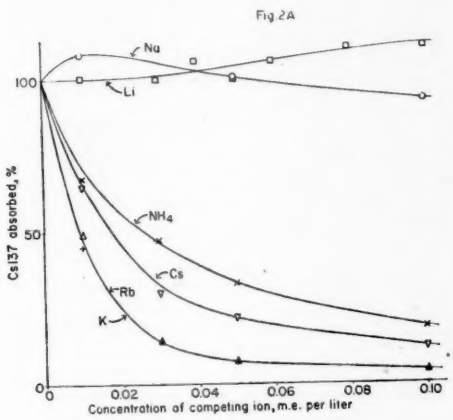
[2] This paper is based on work performed under contract number AT-(11-1)-34, project 23, with the U.S. Atomic Energy Commission.

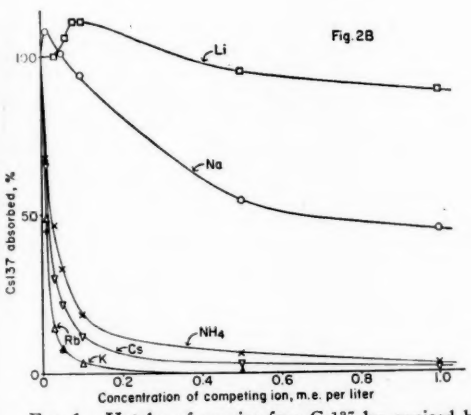
14% of that taken up at 26° C is absorbed at the lower temperature. Whether this small amount is to be ascribed wholly to non-metabolic uptake or at least partly to the persistence of metabolism at 2° C is not determined although the latter possibility seems most likely in view of the very short periods of initial equilibration found by Bange and Overstreet (1) at higher concentrations of Cs. It is clear in any case that the rapid initial uptake commonly observed and attributed to an involvement of the so-called "free space" is relatively not significant here.

The effects upon absorption of Cs^{137} of various monovalent cations over three concentration ranges are shown in figures 2A to 2C. In these graphs the absorption of Cs^{137} is expressed as percent of its absorption in the absence of a competing cation.

It is apparent from the data that at low concentrations Na and Li had no appreciable effect upon the absorption of carrier-free Cs^{137} although the ratios of the concentrations of these ions to that of Cs^{137} in the solutions were of course enormous. The other monovalent cations had large effects over all ranges of concentrations investigated, K and Rb being most effective in lowering the uptake of Cs^{137}. The effect of Li is not surprising in view of the finding of Bange and Overstreet (1) that Li is not absorbed from solutions containing Li at low concentrations. Also, unpublished data from the laboratory of L. Jacobson







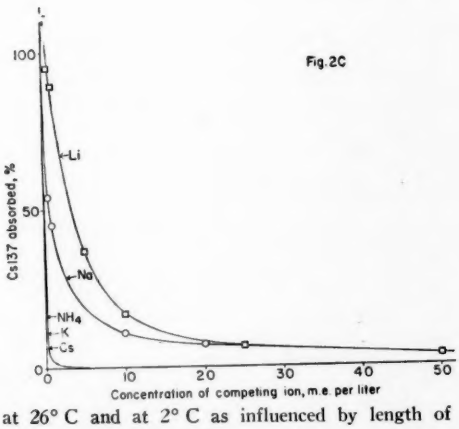


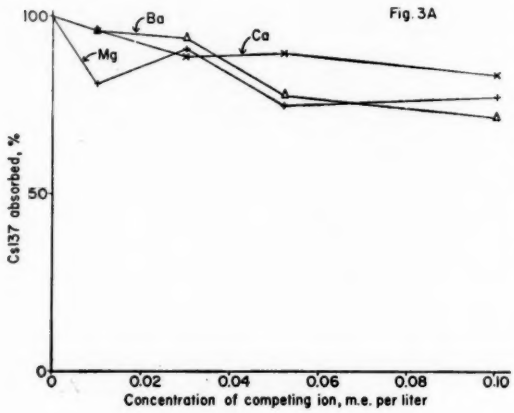
FIG. 1. Uptake of carrier-free Cs^{137} by excised barley roots at 26° C and at 2° C as influenced by length of absorption period. Percentage of Cs^{137} remaining in culture solution as a function of time.

FIG. 2A. Effects of monovalent cations in the concentration range of 0 to 0.10 meq per liter upon the uptake of carrier-free Cs^{137} by excised barley roots. The absorption of Cs^{137} is expressed as percent of its uptake in the absence of a competing cation.

FIG. 2B. Effects of monovalent cations in the concentration range of 0 to 1.0 meq per liter upon the uptake of carrier-free Cs^{137} by excised barley roots.

FIG. 2C. Effects of monovalent cations in the concentration range of 0 to 50 meq per liter upon the uptake of carrier-free Cs^{137} by excised barley roots.

show little or no absorption of either Li or Na by this tissue at solution concentrations of 0.5 me/l or less. K, Rb, and Cs, however, were absorbed in substantial amounts in this concentration range. Thus the data are in accord with the hypothesis advanced by Bange and Overstreet (1) calling for two mechanisms of Cs absorption one of which is most active at low concentrations of this element, the other operative when Cs concentrations are at a higher level and the first mechanism is unable completely to take care of the task of absorption. It is presumably the first mechanism that we are dealing with here. As previously suggested (1) the first mechanism is relatively non-selective since the monovalent ions NH_4, K, and Rb may make use of it. We must suppose from the data, however, that Na and Li may not be absorbed to any extent by this means.



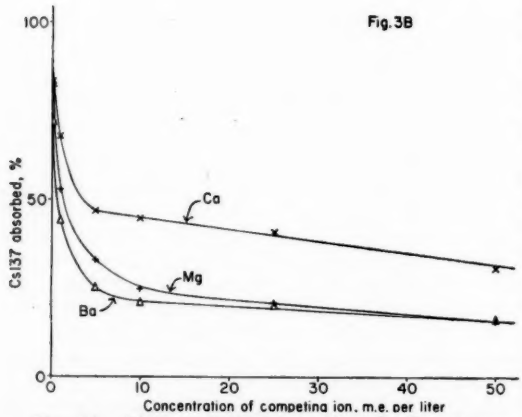


FIG. 3A. Effects of divalent cations in the concentration range of 0 to 0.10 meq per liter upon the uptake of carrier-free Cs^{137} by excised barley roots.

FIG. 3B. Effects of divalent cations in the concentration range of 0 to 50 meq per liter upon the uptake of carrier-free Cs^{137} by excised barley roots.

The increased effectiveness of Na when its concentration is increased may possibly be explained in two ways. First, the mechanism for Na absorption may not be entirely independent of the first mechanism for Cs. The utilization of some precursor common to both mechanisms may at high concentrations of Na reduce the effectiveness of the first Cs mechanism. Second, it is conceivable that adsorption upon fixed negative charges in the protoplasm or elsewhere is a necessary prelude to true metabolic absorption (4). If this is the case, competition for adsorption sites may be partly what is observed at higher concentrations of the competing ions studied here rather than competition for the actual mechanisms of metabolic uptake. The relatively greater effect of Na compared to that exerted by the presumably more strongly adsorbed divalent ions (fig 3B) suggests, however, that the first possibility plays a role.

The effects of three divalent ions upon uptake of Cs^{137} over two concentration ranges are shown in figures 3A and 3B. Like Na and Li, Ca, Ba, and Mg have very small effects upon the uptake of Cs^{137} in the low concentration range. The data obtained with these ions were much more erratic than those found for monovalent ions. The graphs have been drawn with straight lines between the points to emphasize this.

The reason for the rather wide variations encountered when the competing ions are divalent is not clear. In repeated trials this variability was found to be a persistent feature even when possible contamination was scrupulously avoided. In view of the pronounced effect obtained with potassium, it seems most likely that small variations in the initial K content of the root samples may have played a part.

It is interesting to note that the behavior of Cs^{137} with respect to competition with other ions in absorption by this tissue parallels to some extent its behavior in adsorption on soil colloids. Schulz, Overstreet, and Barshad (5) have shown that in five soils studied sodium and divalent ions are much poorer replacing agents of adsorbed Cs^{137} than are K, NH_4, and Cs.

This phenomenon was ascribed to the formation of insoluble crystalline substances whose structure permits isomorphic substitution of K and NH_4 for Cs but not of Na, Mg, or Ba. It has been suggested (3) that essentially the same phenomenon may occur in plant tissues, i.e., component structures of the cytoplasm (carriers?) may require certain ions for greatest stability. Although further speculation is perhaps not warranted at this time, the possibility is an intriguing one. We have one piece of evidence tending to support the idea. It was found in studies with K^{42} and Cs^{137} that the ratio of K to Cs taken into the excised roots was not temperature dependent, K being preferred over Cs at 2° C to the same extent as at 26° C. In view of the higher adsorption energy of Cs, one might have expected a reversal of the relationship with a preference for Cs occurring when

metabolism was lowered drastically at 2° C. These experiments will be reported fully at a later date.

Summary

The effects of the monovalent cations K, Na, NH_4, Rb, Li, and Cs and of the divalent cations Ca, Mg, and Ba upon the absorption of carrier-free Cs^{137} by excised barley roots has been investigated.

In the concentration range of 0 to 0.10 meq per liter Na, Li, Ca, Ba, and Mg had essentially no depressant effect upon Cs^{137} uptake whereas K, Rb, NH_4, and Cs were markedly effective.

At higher concentrations all ions investigated inhibited the absorption of Cs^{137}.

The uptake of carrier-free Cs^{137} was found to be strongly temperature dependent and is therefore largely the result of metabolic absorption rather than of diffusion and adsorption phenomena. In spite of this the behavior of the ions studied in inhibiting uptake of Cs^{137} parallels their behavior in respect to competition for exchange sites upon soil colloids. A possible reason for this is discussed.

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PHOTOSYNTHESIS & RESPIRATION IN DEVELOPING FRUITS[1]. II. COMPARATIVE RATES AT VARIOUS STAGES OF DEVELOPMENT

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Surface layers of the young fruits of many plants contain chlorophyll and apparently are capable of carrying on photosynthesis as was shown by the use of $C^{14}O_2$ in the previous paper of this series (3). It would be of some interest to know to what extent photosynthesis in the fruit might contribute to the growth of the fruit. Little quantitative work has been done on photosynthesis in green fruits. Kursanov (8) made measurements of the photosynthetic rates of apple, cucumber, and pea fruits in sunlight. His data seemed to show the rather surprising effect that photosynthesis in the fruit was markedly increased when the fruit was detached from the plant or the interchange between the plant and the fruit was altered by severing the phloem. It has been estimated from experiments where developing barley ears were shaded that as much as 30 % of the assimilate may be produced by that organ itself (1, 10). However, developing flowers and fruits do put heavy demands on the carbohydrate supply of a plant, as noted for cotton (5).

It seemed desirable to extend the available data on photosynthesis to other fruits. This report presents data for photosynthesis and respiratory rates in lemons, oranges, and avocados of varying ages. For simplicity, these experiments were carried out with detached fruits. Kursanov's suggestion that detaching the fruit leads to drastic metabolic changes made it mandatory to determine if such changes actually occurred in our experiments. Data obtained under better controlled conditions than those used by Kursanov are presented here to demonstrate that marked changes in the photosynthetic or respiratory behavior of the fruit do not occur when the fruit is detached from the plant.

Methods & Materials

For experiments where detached fruits were used, the fruits were contained in large, wide-mouthed Warburg vessels (75 cm^3) immersed in a water bath at 25° C. They were illuminated either from below with fluorescent lamps (approx 1,000 ft-c) or from above with sunlight (approx 10,000 ft-c).

[1] Received revised manuscript June 30, 1960.

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Under these conditions, the photosynthetic rate values were obtained from the formula $PS = A(r-p)$, where PS is photosynthetic rate in CO_2 assimilation per hour, A is the air flow rate factor, r is the measured CO_2 content of the effluent air during respiration in the dark and p is the CO_2 content of the exit air during photosynthesis. Respiration rate (CO_2 evolved/hr) was similarly obtained from $R = A(r-a)$ where A and r are the same as above and a is the CO_2 content of the input air.

Air was passed over each fruit at an accurately determined rate of flow. The rate of flow used for various experiments was as follows:

Oranges, lemons, and avocados (data reported in figures 1, 2, 3) about five liters per hour. Oranges in sunlight and in artificial light (table I, fig 6) about 18 to 23 liters per hour. Oranges in dark (table I & fig 6) about five liters per hour. Cucumbers (table I & fig 5) about 12 liters per hour. The air leaving the flask was passed through a drying tube into a Liston-Becker Infra-red CO_2 Analyzer Model 15 to measure the CO_2 content of the air. The rate of flow was adjusted so that CO_2 measurements were within a readable range on the analyzer. During photosynthesis the content of the air passing out of the flasks containing the fruit was not allowed to fall below 0.02 % CO_2.

For experiments in which fruits remained attached to the plant, individual fruits were inserted into a container of suitable size and a seal was made around the stem with modeling clay. Air was passed through the container and analyzed as described above. The plants were maintained in air at 20° ± 0.5 C. Light was supplied with a bank of fluorescent lamps (approx 1,000 ft-c).

Surface areas of avocado and lemon fruits, which are ellipsoidal, were calculated using the tables compiled by Turrell (9). For oranges, the displacement volume was determined and the surface area calculated with the assumption that the orange was a perfect sphere. Areas of large cucumber fruits were determined by peeling the fruit and measuring the area of the peel with a planimeter.

The fruits used were: Eureka lemon (*Citrus limon*, Burm. f.); Valencia orange (*C. sinensis*, Osbeck); Hass avocado (*Persea americana*, Mill.); Heinz Pickling cucumbers (*Cucumus sativus*, L.).

Chlorophyll content of the fruit surface was determined by extracting the peel with acetone, centrifuging, and diluting to a known volume. The optical density of the supernatant liquid was measured at 665, 644, and 624 mμ with a Beckman Model DU Spectrophotometer and the chlorophyll a concentration was calculated from this information using the formula of Koski (7).

Results & Discussion

Contribution of Photosynthesis to Developing Green Fruit. Lemons could be obtained in widely varying sizes for single experiments for simultaneous comparison of photosynthetic and respiratory activity at different ages, hence data are more extensive for lemons than for the other fruits. It is apparent (fig 1) that photosynthetic activity of the lemon fruit declines gradually with an increase in fruit size. The photosynthetic rates for oranges and avocados appear to follow similar trends for the ranges examined.

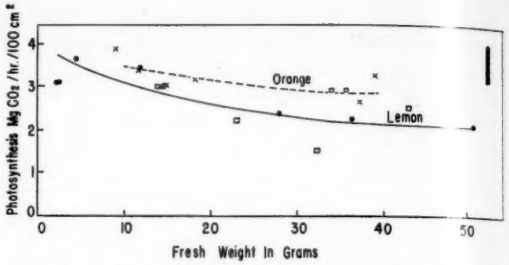
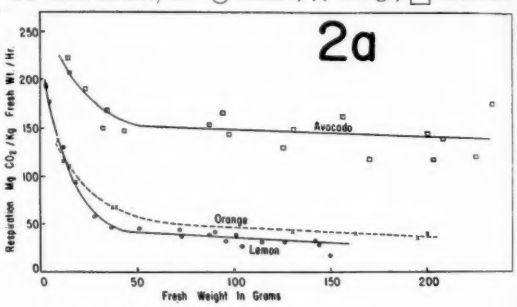
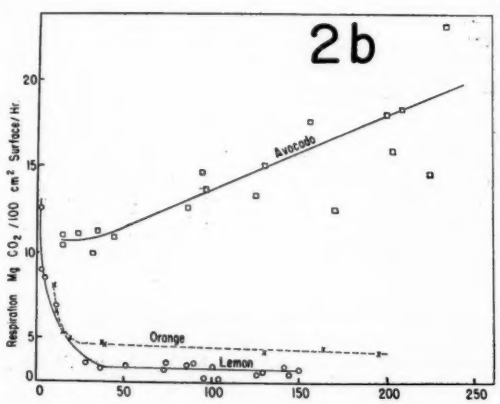


Fig. 1. Relationship of photosynthesis to fruit size. ○ Lemon, × orange, □ avocado.

Contributions of photosynthesis to the growth of the fruit must be examined in conjunction with the

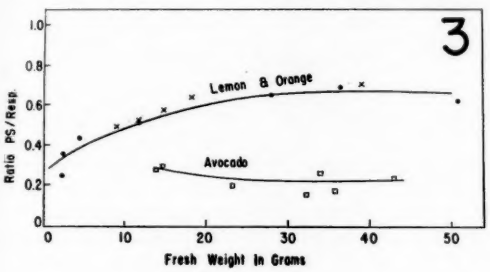
Fig. 2. Relationship of respiration to fruit size. 2a, Respiration/kg fresh weight/hr and 2b, Respiration/100 cm² fruit surface/hr. ○ Lemon, × orange, □ avocado.

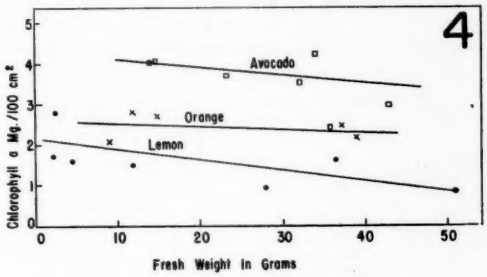


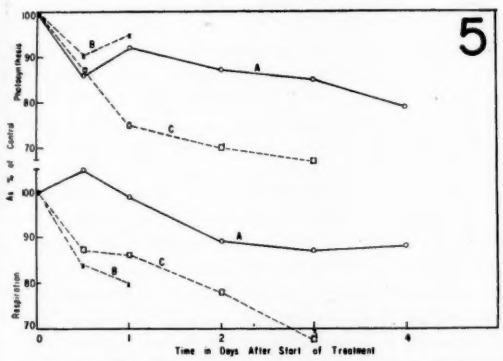


respiratory requirements. Plotted on the usual basis of the CO_2 output per unit weight of fruit (fig 2a), a steep decline occurs during the early growth of the lemon and a slightly lower decline occurs during the growth of avocados and oranges. Bain (2) obtained a similar result for respiration in developing Valencia orange fruit. When respiration is plotted as a function of the surface for lemons and oranges (fig 2b) it becomes apparent that respiration is quite constant, per unit of surface, during the later stages of growth. This would indicate that respiration in the larger citrus fruits is, in some manner, more a function of surface than of the total mass of the fruit. This idea fits well with other data which indicates that the outer flavedo tissues of the citrus fruits carry on respiration at rates many times higher than the underlying tissues (6). Avocados, on the other hand, show a positive slope for the surface plots during most of the growth period, indicating that the surface is not the restricting factor in the respiration of avocado tissues and that the mass of the fruit is of more importance. Presumably, during the early stages of growth, all tissues of the citrus fruits are also active in respiration, giving rise to the bimodal curves. The break in the curve would correspond approximately to the transition from the cell division stage to the cell expansion stage.

Figure 3 clearly shows that the ratio of photosynthesis/respiration for lemons and oranges increased during the early growth stages because of the very rapid decrease in respiration with the slower fall of photosynthesis. Thus for the citrus fruits, photosynthesis may actually be offsetting a greater percentage of the respiratory loss of CO_2 in the large fruit than in the very small fruit. This is of particular interest since the surface area will increase only as the square of the radius while the mass of the fruit will be proportional to the cube; therefore the contribution of photosynthesis per unit of weight in the large fruit might have been expected to be considerably less than in the small fruit. In the later stages of development, the ratio seems nearly constant although it would be expected to drop sharply with the loss of chlorophyll accompanying ripening. It should be noted that photosynthesis, in no case, reached the compensation point. Since the photosynthesis measurements made in the above experiments were made with fluorescent lights of relatively low intensity, in one experiment the rate was determined using sunlight. For fruit weighing 52 grams, the photosynthetic/respiratory ratio reached







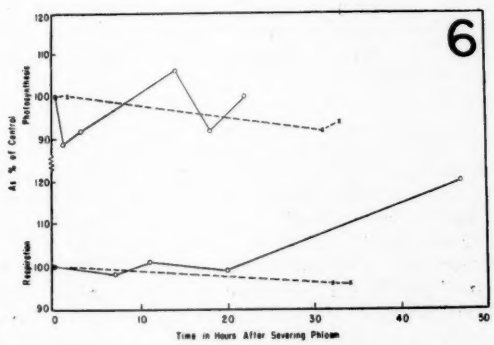


FIG. 3. Relationship of the ratio photosynthesis/respiration to fruit size. ○ Lemon, × orange, □ avocado.

FIG. 4. Relationship of chlorophyll content to fruit size. ○ Lemon, × orange, □ avocado.

FIG. 5. Changes in photosynthesis and respiration of cucumber fruit as the result of treatments affecting food supply to the fruit. A, Phloem severed. B, Completely severed. C. Leaves removed from vine.

FIG. 6. Effect of severing the phloem connecting citrus fruits to the tree on the subsequent rates of photosynthesis and respiration. ○ -- ○ Exp 1; × -- × Exp 2.

0.99 for sunlight (actual photosynthesis was 3.38 mg CO_2/hr/100 cm²) vs. 0.46 in fluorescent light. Thus even with fruit exposed to sunlight and air containing about 0.03 % of CO_2, the contribution of photosynthesis could not do more than supply enough energy to replace that used in respiration while the plant was exposed to the light.

The ratio of photosynthesis to respiration for avocados (fig 3) was considerably lower than for citrus and the only trend observed was that with the smaller fruit there was a slight increase in this ratio. The respiratory rate of these fruit being higher in relation to the rate of photosynthesis would indicate that the contribution of photosynthesis to fruit growth would be of even less significance than for citrus.

The chlorophyll contents of the fruit used for the photosynthesis and respiratory measurements are given in figure 4. The lemon and avocado fruit had a higher chlorophyll a content in the smaller fruit while the orange remained constant over the range sampled. There was not a good correlation between photosynthetic activity and chlorophyll content.

TABLE I

PHOTOSYNTHESIS & RESPIRATION AT 20° C OF ORANGE & CUCUMBER FRUITS WHILE STILL ATTACHED TO PLANT

PLANT MATERIAL	WT/ FRUIT g	SURFACE /FRUIT cm²	RESP mg.CO_2 /kg fr wt/hr	PHOTO. mg.CO_2 /hr/ 100 cm²	RATIO PHOTO/ RESP
Orange fruit	43.14	68.4	40.0	2.50	1.04
	62.05	98.5	38.6	0.66	0.31
Cucumber fruit	2.0	7.0	99.0	2.00	0.71
	6.0	18.0	81.3	1.99	0.73
	22.0	42.0	56.6	1.66	0.56
	26.2	47.0	43.0	2.40	1.00
	61.0	93.0	109.9	4.53	0.63
	67.0	101.0	84.4	5.49	0.65
	90.9	127.0	44.0	1.86	0.59
	124.8	160.0	41.0	1.95	0.61

PHOTOSYNTHETIC & RESPIRATORY EFFECTS AS RESULT OF INTERRUPTING FRUIT'S FOOD SUPPLY: Kursanov (8) reported that cucumbers apparently did not photosynthesize at all when still attached to a normal plant, but when leaves were either covered with bags, or the fruit removed from the plant, photosynthesis in the fruit rose sharply from the initial level of zero uptake of carbon dioxide to a value as high as 5.3 mg CO_2/100 cm²/hr. In re-examining these results by measuring photosynthesis in attached and detached oranges and cucumbers we found photosynthetic rates ranging from 1.7 to 5.5 mg CO_2/100 cm²/hr for cucumbers attached to normal plants (table I). Oranges were also found to carry on significant photosynthesis while still attached to normal plants (table I). The results here should not be directly compared with the detached fruit experiments reported in figure 1 since they were done at different temperatures, but the photosynthesis/respiration ratios are in similar ranges (fig 3 & table I), suggesting that there is no essential difference between attached and detached oranges.

The comparative effects of severing the phloem of the fruit stem, removing the leaves from the plant, or completely severing the fruit are illustrated in figure 5 for cucumbers and in figure 6 for oranges. Each fruit was compared with a normal fruit attached to a normal plant over a period of time following the treatment. For all treatments cucumbers show a relatively steady drop in both photosynthesis and respiration following treatment while oranges show little or no significant changes in either factor during our experimental period. Other workers (4) have found that there is generally a gradual decrease in respiration of citrus fruits after detaching them from the tree.

From this data we may conclude that in cucumbers and oranges, photosynthesis and respiration occur at comparable rates in the fruits attached to normal plants or in freshly detached fruits. After detaching, the rates of photosynthesis and respiration appear to decline slowly.

SUMMARY & CONCLUSIONS

Measurements were made on rates of photosynthesis and respiration in green orange, lemon, and avocado fruits of various ages and sizes, ranging from 2.5 g to over 200 g. Photosynthetic activity, per unit of surface declined somewhat during growth in lemons and oranges although little or no change was noted for avocados. Respiration in citrus drops rapidly during early growth causing an increase in the photosynthesis/respiration ratio in the larger fruit. In avocados there was little change in this ratio and the ratio was considerably smaller than for citrus. In no case did the photosynthetic rate of the various fruits exceed the compensation point and in fluorescent light, the rate of photosynthesis was well below the compensation point. It is concluded that the contribution of photosynthesis within a fruit towards its own development is small.

The respiratory rate calculated on a surface basis remained almost constant for citrus fruit in the range of 30 to 200 g while the respiration of avocados per unit of surface showed a marked increase with an increase in fruit size. This would indicate that respiration in the larger citrus fruits is more a function of surface than of the total mass of the fruit, while the reverse is true for avocados.

The effect on photosynthesis of interrupting food supply to the fruit was also examined. Using cucumbers and oranges, photosynthesis was measured with fruit attached to normal plants, attached to normal plants but with phloem of the fruit stem severed, attached to defoliated plants, or with the fruit com-

pletely detached. In contrast with an earlier report that similar treatments gave rise to large increases in photosynthetic rates in cucumbers, little or no stimulation of respiration or photosynthesis was produced by any of these treatments. Instead, there appears to be a gradual decline in both photosynthesis and respiration.

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INFLUENCE OF COBALT ON NITROGEN FIXATION BY MEDICAGO [1]

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Reisenauer (6) has discussed the reasons for exploring the possibility that cobalt may be implicated in symbiotic nitrogen fixation. Data were also presented which demonstrated the essential role of cobalt in the functioning of the Rhizobium-alfalfa (*Medicago sativa* L.) system in nitrogen fixation.

The present paper presents additional data obtained during the early winter season (Oct.–Dec. 1959) under somewhat more favorable greenhouse conditions and with some other modifications. As a consequence yield differences reflecting efficiency of nitrogen fixation with and without cobalt supplements are considerably greater than those reported earlier for summer (May–July 1959) experiments, as well as those obtained by Ahmend and Evans with *Glycine max* Merr. (1). Also, numerous requests for more detailed information on preparation of growth media sufficiently low in cobalt to permit observations of direct positive influence of the element on nitrogen fixation make it seem desirable to elaborate in detail on our experimental procedures in this paper.

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Materials & Methods

Water Supply: All water used in cleaning glassware, including solution storage bottles and culture vessels, in preparing nutrient media, and in making up water during the growth period was of high purity as determined by the dithizone test for heavy metals. Distilled water prepared by the usual procedures in an all block-tin system was again distilled from a still provided with a borosilicate glass head and condenser. Water was stored in borosilicate glass bottles.

Glassware: All glassware, of borosilicate glass, was rigorously cleaned by washing with 0.1 M trisodium ethylenediamine tetraacetic acid (EDTA) solution, rinsing with redistilled water, followed by washing with 1 M HNO_3, and rinsing again with redistilled water. Culture vessels were 4-liter beakers with covers of plaster of paris coated with paraffin.

Purification of Salts Used in Nutrient Solutions. Earlier experiments designed to test the cobalt requirements of tomatoes indicated that cobalt could be successfully removed from salt solutions by alkaline sulfide precipitation with copper as carrier (3). However, further study of the procedure

(Frinck, C. R. MS thesis U.C., 1957) cast doubt on the general reproducibility of these purification procedures. Therefore other procedures were investigated, using Co^{60} as an indicator of efficiency of purification.

The method adopted for these experiments was based on coprecipitation with iron oxinate as described by Hewitt (5) with some modification.

Volatile acids, ammonia, and organic solvents were distilled before use. Solutions of 2 M $Ca(NO_3)_2$, 1 M K H_2PO_4, and 0.5 M K_2SO_4 were prepared. Twenty milliliters of a 5% oxine solution in 2 M acetic acid and 5 ml of 0.2 M $FeCl_3$ were added to each liter of salt solution. Ammonium hydroxide (approx 6 M) was added to each solution to a pH of 5.1 to 5.2. After standing overnight, the solution was filtered through thoroughly washed filter paper and the precipitation step repeated. Excess oxine was extracted from the second filtrate with chloroform. Finally the chloroform was removed from the solutions by several extractions with diethyl ether. Residual ether was removed by heating the solutions on the steam bath.

Calcium sulfate was prepared by mixing equimolar quantities of the purified calcium nitrate and potassium sulfate. The precipitate was washed with redistilled water until free of nitrate as shown by a diphenylamine test of the washings.

Calcium carbonate was prepared by passing carbon dioxide and ammonia gas into a solution of purified calcium nitrate, keeping the pH in the 8 to 9 range. The precipitate was freed of nitrate by washing with redistilled water.

Magnesium sulfate was purified by extracting a 1 M solution at pH 8.5 with 0.01% dithizone in carbon tetrachloride. Three successive 100 ml portions of the dithizone solution were used to extract the heavy metals from 1 liter of magnesium sulfate. If the magnesium sulfate contains large amounts of dithizone-extractable metals more extractions may be necessary. Excess dithizone was removed from the aqueous magnesium sulfate solution by repeated extractions with chloroform. Soluble chloroform was removed by extracting with diethyl ether and the ether was removed by heating on the steam bath.

Iron was freed of cobalt by extracting a $FeCl_3$-6 M HCl solution with diethyl ether which had been saturated with 6 M HCl. As shown by Grahame and Seaborg (4) with radiocobalt and an iron to cobalt ratio of 10^8 : 1, separation of iron and cobalt is quantitative. The ether and excess HCl were removed by evaporation of the solution to a dense syrup on the steam bath. The $FeCl_3$ was made to standard volume and an aliquot analyzed for iron prior to preparation of the chelated micronutrient solution.

Other micronutrients: Manganese chloride and the sulfates of zinc and copper were twice crystallized. Boron was supplied from twice crystallized boric acid. Chloride requirement for plants was met by the chloride associated with ferric chloride and manganese chloride.

TABLE I

MICRONUTRIENT STOCK SOLUTION

SALT	1 ml STOCK/L OF CULTURE PROVIDES FOLLOWING CONCS OF ELEMENTS		
	ELEMENT	μg ATOMS/L	PPM APPROX
H_3BO_3	B	25	0.27
$MnCl_24H_2O$	Mn	5	0.27
	Cl	10	0.36
$ZnSO_47H_2O$	Zn	2	0.13
$CuSO_45H_2O$	Cu	0.5	0.03
Na_2MoO_4	Mo	0.5	0.05
$FeCl_3$	Fe	90	5.0
	Cl	270	9.5
EDTA	...	97.5	...

EDTA was purified by dissolving the acid in dilute ammonium hydroxide and reprecipitating it by the addition of HCl. This procedure was repeated twice. The acid was dried under vacuum at 70° C for 24 hours to provide the anhydrous form.

Chelated micronutrient stock: The micronutrient solution was prepared in such concentration that 1 ml per liter of culture solution provided the concentration of micronutrients indicated in table I. Sufficient EDTA was used to provide an equimolar ratio with the micronutrient cations. Redistilled ammonium hydroxide was added to neutralize the acidity of the EDTA and $FeCl_3$ to a final pH of 5.5. The solution was stored in a dark bottle at 4° C.

The concentrations of macronutrients are given in table II. One-fourth of the indicated concentration was present in the solution at the time of planting the seedlings. After 10 days the remainder was added. In addition, all treatments received an initial application of 1.25×10^{-4} moles of $Ca(NO_3)_2$ per liter to provide a limited amount of nitrogen until symbiotic nitrogen fixation became effective. When differential treatments were applied (10 days after planting)

TABLE II

MACRONUTRIENT SOLUTION COMPOSITION

SALT SOURCE	mg ATOMS OF ELEMENT/L NUTRIENT				
	Ca	Mg	K	S	P
KH_2PO_4	...	...	2	...	2
K_2SO_4	...	...	4	2	...
$MgSO_4$	...	1	...	1	...
$CaCO_3$	2	...	...	...	...
$CaSO_4$	2	...	...	2	...
Total mg atoms/l	4	1	6	5	2
Total ppm	160	24	235	160	62

two sets of plants were placed on a high nitrogen regime receiving 5×10^{-3} moles of $Ca(NO_3)_2$ per liter of culture (see table III). $CaCO_3$ and $CaSO_4$ were not added to these beakers because calcium was supplied by the $Ca(NO_3)_2$ addition.

Solutions were not changed during the 8-week experimental period. Occasional checks of pH of the nutrient were made with pH values ranging from 6.0 to 6.5. No adjustment of pH was made.

Alfalfa seedlings, germinated on cheesecloth in 1/20 strength nutrient solution in glass trays, were transferred to the culture vessels when 10 days old. The culture vessels were covered with double thickness jackets (blue denim inside, white outside) to exclude light from the solution. Continuous aeration was employed.

INOCULATION: Week-old cultures of *Rhizobium meliloti* from mannitol-yeast extract agar plates were used in preparing the suspension for inoculation. Removal of possible cobalt contamination in the mannitol-yeast extract agar media was not attempted. Reliance was placed on the high order of dilution of the microorganisms with high purity water in minimizing cobalt contamination. One milliliter of a suspension containing approximately 10^4 organisms was added to each 4-liter culture 10 days after the plants were placed in the solutions. Two weeks later, because nodulation was very sparse, an additional inoculation of approximately 10^6 organisms per 4 liters was made. Within a week nodulation was profuse.

TRACER STUDIES: For an experiment with isotopic nitrogen, excised nodulated roots from treatment A (minus cobalt) and from treatment B (plus cobalt) were cut into approximately 3-cm lengths and 5-g samples of each were added to Thunberg tubes. Samples were incubated in the presence of isotopic nitrogen gas. After a suitable incubation period the root tissue was subjected to Kjeldahl digestion and N^{15} was determined on the ammonium ion obtained therefrom by means of a ratio-type mass spectrometer.

TABLE III

DIFFERENTIAL TREATMENTS

TREATMENT DESIGNATION	VARIABLES		RHIZOBIUM INOCULATION
	NITRATE CONC	Co (0.1 μmoles/l)	
A	1.25×10^{-4} M	−	+
B	1.25×10^{-4} M	+	+
C	1.25×10^{-4} M	+	−
D	1.25×10^{-4} M	−	−
E	51.25×10^{-4} M	−	−
F	51.25×10^{-4} M	+	−

EXPERIMENTAL DESIGN

Three beakers of 4-liter volume, each containing 5 alfalfa (*Medicago sativa*) plants, were used for each of six treatments. All treatments had an initial supply of nitrate nitrogen of 1.25×10^{-4} moles per liter. Ten days after transfer of seedlings to the 4-liter beakers the differential treatments were imposed. Treatments are shown in table III.

RESULTS

Treatments of principal interest are A and B, Rhizobium inoculated without and with supplemental cobalt, respectively. Treatments C and D were included to observe the extent of nitrogen deficiency obtaining during the growth period with the restricted fixed nitrogen supply (1.25×10^{-4} M) and no intentional inoculation with Rhizobium. The yields (table IV) obtained in these treatments served as reference points for judging the effect of cobalt on nitrogen fixation in inoculated systems. One beaker of treatment C was accidentally inoculated the second time. The error was noted immediately and this beaker was then designated as a treatment B. Thus treatment B

TABLE IV

EFFECT OF COBALT & RHIZOBIA ON GROWTH OF ALFALFA

TREATMENT*	FRESH WT YIELD—GRAMS PER CULTURE			
	TOPS		ROOTS	
	MEAN WT	S_x**	MEAN WT	S_x
A Nodulated — no cobalt	12.7	0.569	38.0	1.05
B Nodulated + cobalt	42.5	2.17	36.2	1.69
C Non-inoculated + cobalt	10.1	0.90	33.0	1.50
D Non-inoculated — no cobalt	11.1	0.292	32.7	1.11
E $Ca(NO_3)_2$ — no cobalt	101.2	8.94	96.3	14.1
F $Ca(NO_3)_2$ + cobalt	96.3	5.07	92.0	13.0

* All beakers received an initial supply of nitrate, at the rate of 1.25×10^{-4} moles per liter. Treatments E and F received additional Ca $(NO_3)_2$ at rate of 5×10^{-3} moles per liter 10 days after the start of the experiment. Plus cobalt treatments received 0.1 μmoles $CoSO_4$ per liter of cultures.

** Standard error of mean.

then consisted of four beakers and treatment C of only two. No nodules were found on plants which were not inoculated.

Treatments E and F are of interest only in reference to the effect of cobalt supplements on yield of alfalfa when supplied with fixed nitrogen. As will be noted later, no specific differences between these two treatments were detected beyond the limits of experimental variations.

Comparisons of yields from beakers supplied with nitrate nitrogen (treatments E & F) with those of beakers where symbiotic nitrogen fixation was operative (treatment B) cannot be made because of difficulties in timing of application of nitrate nitrogen to coincide with initiation of symbiotic nitrogen fixation. It had been planned to add nitrate at the time of appearance of first nodules. However, failure of the first inoculation to provide a reasonable number of nodules required a second inoculation with consequent time delay. Thus, plants of treatments E and F made considerably greater growth than those of treatment B because of a nearly 3-week earlier supply of nitrogen in excess of that provided initially.

A summary of chronology of the experiment may be useful in this regard. Seedlings germinated October 5, 1959; transplanted to dilute nutrient (including 1.25×10^{-4} moles $Ca(NO_3)_2$/liter) on October 15, 1959; full complement of nutrient salts added (except $Ca(NO_3)_2$) to all treatments October 26, 1959; 5×10^{-3} moles $Ca(NO_3)_2$ per liter added to treatments E and F October 26, 1959; $CoSO_4$ (1×10^{-7} moles/liter, approx 0.006 ppm Co) added to plus cobalt treatments October 26, 1959; first inoculation with 10^4 cells of *Rhizobium meliloti* October 26, 1959; second inoculation with 10^6 cells of *Rhizobium meliloti* November 9, 1959; nodulation profuse and green color returning to nitrogen deficient plants of treatment B by November 16, 1959; harvested all plants December 11, 1959.

Note that plants of treatments E and F never became nitrogen deficient because of supply of $Ca(NO_3)_2$. Plants of all other treatments showed clear symptoms of nitrogen deficiency by November 13, 1959. The plants of treatment B regained their green leaf color by November 16, 1959. All plants of other treatments remained nitrogen deficient as judged by pale green leaf color and purpling of stems. Dry weights, nitrogen data and top-to-root ratios are presented in table V. No dry weight figures are available for the roots of treatments A and B, inasmuch as the tissues were utilized for studies with isotopic nitrogen.

It was desirable to determine whether or not there was any directly demonstrable difference in the nitrogen fixing ability of nodulated roots grown in the presence and in the absence of cobalt, as well as to observe the effect on nitrogen fixation of adding cobalt to previously deprived roots and nodules. Nodulated root sections were placed under isotopic nitrogen in Thunberg tubes as indicated in table VI. Samples were incubated for various times as shown in the table and isotopic nitrogen was recovered in the ammonia fraction from Kjeldahl digestion of these tissues. Tissues grown in the presence of added cobalt ion were significantly more active in their capacity for nitrogen fixation. Moreover, tissues grown in the absence of added cobalt ion were able to fix nitrogen provided cobalt was added immediately before the period of administration of isotopic nitrogen. Adding glucose appeared to have little if any effect on accelerating the fixation of nitrogen. It is evident by reference to the various time treatments that most of the nitrogen fixed by the excised root segments was fixed during the first 4 hours of incubation. This finding is in keeping with the observation of Aprison and Burris (2) that the capacity for nitrogen fixation by excised nodules of Glycine fell off rapidly with time after the nodules were removed from the plant.

Discussion

From the above data (tables IV & V) it can be seen that the combination of cobalt and an inoculum of Rhizobium make it possible for plants of *Medicago sativa* to grow luxuriantly under conditions which

Table V

Dry Weights & Nitrogen Values

a Treatment*	Tops				Roots				h Top-root ratio d/g
	b Mean dry wt g	c Mean N content mM/g	d Nitrogen content b x c	S_x	e Mean dry wt g	f Mean N content mM/g	g Nitrogen content e x f	S_x	
A	3.0	0.78	2.34	0.27	**	**	**	...	...
B	7.3	2.79	20.4	0.34	**	**	**	...	...
C	2.3	0.93	2.14	0.28	4.7	0.73	3.44	0.40	0.62
D	2.5	0.92	2.30	0.05	4.9	0.70	3.44	0.20	0.67
E	18.9	1.83	34.6	2.4	14.1	1.00	14.1	2.8	2.45
F	18.0	1.95	35.1	2.1	12.5	1.50	18.7	2.1	1.78

* See table IV.
** Not available. Fresh tissues were used for N^{15} studies.

TABLE VI

FIXATION OF NITROGEN BY NODULATED ROOT SEGMENTS

TUBE NO.	ROOT MATERIAL	INCUBATION TREATMENT	HR INCUBATION WITH N^{15}	TOTAL N (μmoles)	N^{15} CONTENT (ATOM % EXCESS)	N FIXED (μmoles)
1	Treatment A (minus cobalt)	No added cobalt	24	145	0.003	0.0043
2	"	" " "	24	143	0.003	0.0043
3	"	2×10^{-8} Molar $CoSO_4$	24	146	0.018	0.026
4	"	4×10^{-8} " "	24	119	0.016	0.018
5	"	6×10^{-8} " "	24	152	0.016	0.024
6	"	10^{-7} " "	2	120	0.010	0.012
7	"	" " "	4	170	0.010	0.017
8	"	" " "	8	182	0.010	0.018
9	"	" " "	24	185	0.011	0.020
10	"	" " "	48	186	0.011	0.020
11	Treatment B (plus cobalt)	" " "	2	576	0.014	0.081
12	"	" " "	4	496	0.026	0.129
13	"	" " "	8	572	0.036	0.206
14	"	" " "	24	440	0.035	0.154
15	"	" " "	48	554	0.036	0.198
16	"	" " " (no added glucose)	24	477	0.048	0.226
17	"	" " " (no added glucose)	11	572	0.035	0.200

Except as indicated above each tube received 5 grams nodulated root segments, 1 ml 1 % glucose, 10 ml cobalt-free culture solution (treatment A, table IV) and 1 ml 10^{-6} M $CoSO_4$. Tubes were evacuated and refilled with 2 cm 100 atom % N^{15} (40 micromoles) and 50 cm O_2 (1 millimole).

otherwise would produce severe nitrogen deficiency. Absence of either cobalt or Rhizobium inoculum resulated in severe nitrogen deficiency. It should perhaps be pointed out that the initial provision of 1.25×10^{-4} moles of calcium nitrate per liter of culture solution was necessary in order to obtain experimental material with which to work. In spite of this the quantitative figures in terms of fresh weight yield of plant material as a result of the addition of cobalt are striking, with greater than a threefold increase in yield during the 4-week nitrogen stress period. From a qualitative standpoint the difference between plants was even more noticeable. This is due in a large part to the fact that there was essentially no observable difference in the amount of root material produced under the various test conditions (excepting for the greater yield of roots obtained from those plants furnished with additional calcium nitrate (treatments E & F) as explained above. This relationship is further borne out by an inspection of the data given in table V showing nitrogen values and top-to-root ratios resulting from the various treatments.

Not expressed by these data but observable visually as the experiment progressed, was an apparent tendency of the plants to overcome to a limited extent the nitrogen deficiency resulting from a lack of cobalt. There appeared to be a slight greening of the cobalt deficient plants just prior to harvest. There are a number of possible explanations for this observation, including the possibility that a small but significant quantity of cobalt was being added with replacement water. It is also possible that nodulated plants have the ability to fix nitrogen independent of the presence of added cobalt, after a lag period.

Although the above data clearly indicate a dependence of the fixation reaction upon cobalt ion, it is not clear where that ion has its effect in the physiological and biochemical chain of events leading finally to the production of fixed nitrogen in the plant. There is some evidence, circumstantial in nature, which suggests that the presence or absence of cobalt did not influence the growth of Rhizobium nor the process of nodulation, but was more directly concerned with the nitrogen fixation reaction itself, or with the synthesis of enzymes or co-enzymes necessary for that reaction. This is based in part on the observation that there was extensive nodulation, and presumably therefore growth of Rhizobium, in the minus cobalt treatments. No attempt was made to study the nodules in section nor to determine the number of organisms present in them. Perhaps the strongest argument favoring the postulation of a direct role of cobalt in the fixation reaction is the immediate effect which adding cobalt had on the fixation of isotopic

nitrogen by excised nodulated root sections.

The studies with excised root sections also indicate that the capacity for fixation is lost soon after the roots are removed from the plant. This suggests that some metabolite produced in the tops is essential for fixation.

Summary & Conclusions

I. Alfalfa plants (*Medicago sativa*) deprived of sufficient inorganic nitrogen were grown in the presence and absence of added cobalt ion and an inoculum of an appropriate strain of Rhizobium.

II. With added cobalt ion and a Rhizobium inoculum, significantly greater yields were obtained than those observed in the absence of such treatment.

III. Cobalt ion alone or a Rhizobium inoculation alone produced no such enhanced growth over control treatments.

IV. The effect of such treatment on root development was negligible during the period of the experiment, but large differences in top growth were observed.

V. Using isotopic nitrogen, roots and nodules grown in the presence of added cobalt possess a greater capacity for nitrogen fixation than those grown without added cobalt.

VI. When cobalt-deficient nodulated root sections were placed in culture solutions containing as little as 2×10^{-8} molar cobalt ion, nitrogen was fixed within a short period.

VII. With isolated root sections carrying nodules, most of the nitrogen was fixed within the first 2 hours of incubation.

VIII. It is concluded that cobalt ion plays a major role in the Medicago-Rhizobium symbiosis.

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CYTOCHROME SYSTEM IN SCHIZOPHYLLUM COMMUNE[1, 2]

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INTRODUCTION

The wood-rotting basidiomycete *Schizophyllum commune* has been of special interest to mycologists since Kniep first outlined the complex pattern of tetrapolar sexuality in its reproductive cycle. In subsequent studies, a great deal of information has been accumulated on the genetics of this organism (16), but relatively little is known about its intermediary metabolism. Wessels (24) has recently demonstrated the operation of the tricarboxylic acid cycle in the breakdown of carbohydrate by *S. commune*. The present report deals with the respiratory system in *S. commune*, with special reference to the mechanisms by which electrons are transferred to oxygen. Although this aspect of metabolism has been studied in great detail with yeast, very little has been done with the basidiomycetes. Experiments carried out with both the intact vegetative mycelium and with cell-free extracts indicate that respiration is mediated by a classical cytochrome-cytochrome oxidase system.

MATERIALS & METHODS

Schizophyllum commune, Fr., strain no. 699, haploid mating type $A^{41}B^{41}$, was grown at 27° C on a solid, minimal medium containing (per liter of distilled water): glucose, 20 g; $(NH_4)_2HPO_4$, 1.5 g; thiamine hydrochloride, 120 μg; KH_2PO_4, 0.46 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Difco agar, 20 g. In order to follow the growth rate under these conditions, a circular disc (3.5 mm diam) of mycelium was placed as an inoculum on agar in the center of a petri dish, which was then incubated at 27° C and 60 % relative humidity; the diameter of the colony was used as the index of total growth (4). Cultures of mycelial pellets were also grown in aerated liquid media of the same composition, and in this case a macerated mycelial mat was used as the inoculum.

Respiratory rates of the intact mycelium on agar were determined in 3 ml volumetric respirometers by a method developed for slices of plant tissues (21). Mycelium-agar discs (13 mm diam) were removed from the centers of appropriate colonies and the oxygen consumption measured for 1 hour at 25° C. The effects of inhibitors were studied by immersing the discs in buffered inhibitor solutions for 30 minutes and then measuring the respiration. Respiratory rates of the mycelial pellets were followed at 30° C using standard manometric techniques (22). The 72-hour cultures were harvested by centrifugation and the pellets washed with 0.05 M phosphate buffer (pH 7).

To prepare cell-free extracts, the washed mycelial pellets were hand ground with sand in a medium containing 0.05 M phosphate buffer (pH 7) and 0.5 M sucrose. All manipulations were carried out at approximately 4° C. The homogenate was centrifuged at 2,000 × *g* for 10 minutes to remove the abrasive and unbroken filaments. The remaining supernatant was centrifuged at 10,000 × *g* for 20 minutes, and the residue washed by centrifugation and finally suspended in a small volume of isolating medium; this suspension of particles is referred to as the washed mitochondrial fraction, M_w. In an alternative procedure for preparing cell-free extracts, pellets suspended in 0.1 M phosphate buffer (pH 7) were exposed to sonic oscillation (Raytheon, 10 kc) for 15 minutes. After preliminary centrifuging at 2,000 × *g* (10 min) and 10,000 × *g* (20 min), the desired particles were sedimented at 78,410 × *g* for 90 minutes in a preparative ultracentrifuge and finally suspended in the phosphate buffer.

Enzyme activities were followed spectrophotometrically: cytochrome c oxidase and cytochrome c reductase were measured at 550 mμ, and the oxidation of reduced pyridine nucleotides (DPNH, TPNH) at 340 mμ. Organic acid oxidations were measured by standard manometric techniques at 30° C. All reaction vessels included the following cofactors (μmoles): $MgSO_4$, 0.5; cytochrome c, 0.01; diphosphothiamine (DPT), 0.2; coenzyme A (CoA), 0.1; adenosine diphosphate (ADP), 3. Spectrophotometric observations on the turbid particle suspensions were made with a Cary recording spectrophotometer (model 14–M).

RESULTS

RESPIRATION OF MYCELIUM ON AGAR: Since most laboratory manipulations of *S. commune* are carried out with mycelium growing on solid agar medium, the respiratory characteristics were first determined under these conditions. Figure 1 shows the rates of oxygen consumption by discs of mycelium

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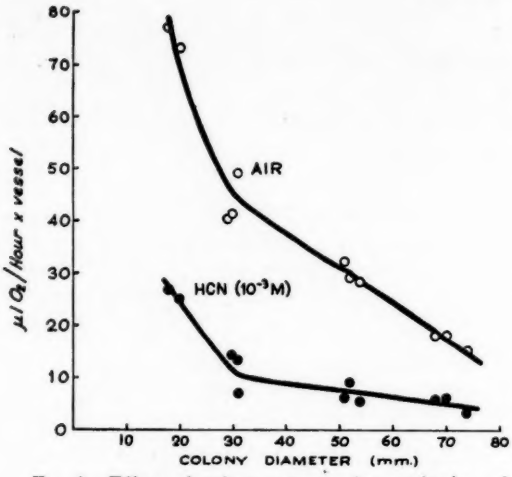


FIG. 1. Effect of culture age on the respiration of discs removed from the center of mycelium on agar. Colony diameters of 20, 30, 50, and 70 mm correspond to 2, 3, 5, and 7 days of growth.

which had been cut out from the centers of colonies of various sizes. The youngest colonies, which had grown for 2 days and reached a diameter of 20 mm, showed the highest respiratory rates (80 µl O_2/hour/vessel containing 2 discs); this rate decreased roughly fivefold after 7 days. A similar rapid decline in respiratory rate takes place during growth of the filamentous fungus *Myrothecium verrucaria* (8). The data of figure 1 also show that the oxygen uptake is markedly inhibited by 10^{-3} M cyanide at all stages of growth examined.

The effects of other respiratory inhibitors were studied using mycelium obtained from colonies of 30 to 40 mm diameter. Sodium azide (10^{-3} M) inhibited the respiration 85 %. Oxygen uptake in the dark was inhibited 45 % by a 95 % CO-5 % O_2 gas mixture, relative to the appropriate N_2/O_2 control. The relative affinity of the oxidase for O_2 and CO, K, calculated according to Warburg's formula (23), varied from 21 to 23. The fact that the CO-inhibition is essentially completely reversed by light (fig 2) strongly suggests that the oxygen-activating enzyme is cytochrome oxidase. The mycelium showed a typical response to the uncoupling agent 2,4-dinitrophenol (DNP): respiration was stimulated 54 % by 10^{-4} M and inhibited 50 % by 10^{-3} M DNP. In parallel studies on the growth of a number of *S. commune* strains, it was found that 10^{-4} M DNP inhibits radial growth approximately 85 %. These results indicate that in *S. commune*, as in other aerobic organisms, interference with oxidative phosphorylation disrupts both the respiratory control and the energy supply for growth.

RESPIRATION OF MYCELIAL PELLETS: The respiratory characteristics of the mycelium were also

TABLE I

EFFECTS OF INHIBITORS ON RESPIRATION OF MYCELIAL PELLETS OF SCHIZOPHYLLUM COMMUNE*

INHIBITOR	CONC (FINAL)	% INHIBITION
Cyanide	0.001 M	85
Azide	0.001 M	85
Carbon monoxide (dark)	$CO/o_2 = 19/_1$	50
DIECA	0.001 M	5
PTU	0.001 M	6
Antimycin A	1 µg/ml	95
Phenylmercuric acetate	0.0001 M	97

* The cyanide mixtures were those recommended by Robbie (17). For the CO experiments, control vessels were flushed with a N_2/o_2 ($19/_1$) gas mixture. All the inhibitors were studied at pH 7 with the exception of azide, which was studied at pH 5.

studied using pellets harvested from liquid cultures after 3 days of aerobic growth. These pellets consumed oxygen at a constant rate (5–8 µl O_2/hr/mg dry wt) for more than 90 minutes. The rates were directly proportional to the amount of mycelium added to the Warburg vessels and they were not enhanced by displacing the air in the gas phase with oxygen.

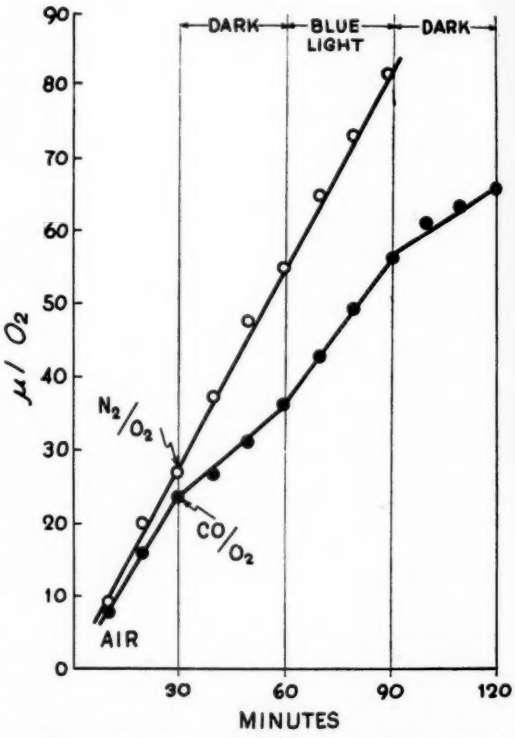


FIG. 2. Effect of carbon monoxide on the respiration of mycelium on agar.

No attempt was made to reduce this endogenous respiration to a point where stimulations by glucose could be demonstrated.

The nature of the respiratory system was examined with the aid of specific inhibitors, and the results are summarized in table I. Oxygen uptake was reduced 85 % by either 10^{-3} M cyanide or azide. In close agreement with the results obtained with the mycelium grown on agar, a 95 % CO-5% O_2 mixture inhibited pellet respiration 50 % in the dark; the 95 % N_2-5 % O_2 control was inhibited 40 %, relative to the rate in air. Millimolar concentrations of two copper chelating agents, diethyldithiocarbamate (DIECA) and phenylthiourea (PTU), inhibited respiration only slightly. Since it is known that these reagents are powerful inhibitors of polyphenol oxidase (12), it would appear that this enzyme, which is widely distributed in the basidiomycetes, does not play a significant role in normal respiration. The respiration was very sensitive to antimycin A, which blocks electron transfer between cytochrome b and c (6), as 1 μg/ml antimycin A (applied in 95 % ethanol) reduced the oxygen uptake to 5 % of the control (containing an equivalent amount of alcohol, which was not itself inhibitory). Phenylmercuric acetate (10^{-4} M), which combines with sulphydryl groups, also inhibited the respiration almost completely. The effects of a wide range of DNP concentrations are shown in figure 3: at low concentrations the oxygen uptake was stimulated as much as 50 %, while high concentrations were inhibitory.

Activities of Isolated Particles: Since it is generally agreed that the respiratory chain in aerobic organisms, including the fungi (7), is localized in mitochondria, special attention was focused on the particulate fraction (M_W) isolated from homogenates of hand-ground mycelium. When particles were added to a reaction mixture containing reduced cytochrome c, the optical density at 550 mμ decreased rapidly due to the oxidation of this component (fig 4). (Preliminary studies showed that this cytochrome c oxidase activity decreased 40 % when particles were stored in the frozen state for 1 day, and all subsequent work was carried out with freshly prepared particles.) The rate of oxidation was proportional to the volume of M_W employed and there was no reaction when boiled particles were used. The enzymatic activity was inhibited 80 % by 10^{-3} M azide and 70 % by 10^{-5} M KCN; when the cuvette was flushed in the dark with a 95 % CO-5% O_2 mixture the rate of oxidation was reduced to 50 % of the control rate (N_2/O_2 = 19). All these results indicate that there is a typical cytochrome c oxidase on the particles.

The mitochondrial fraction was also able to oxidize DPNH (fig 5). The rate of DPNH oxidation, which was very slow in the air-saturated buffer medium (0.020 μmoles/min × mg protein), was greatly stimulated by adding 2×10^{-5} M cytochrome c (0.179 μmoles/min × mg protein). With cyanide included in the reaction mixture, it was possible to demonstrate

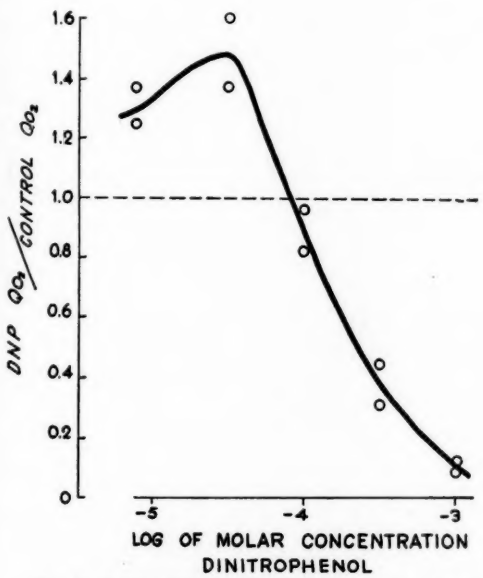


Fig. 3 (*left*). Effects of 2,4-dinitrophenol on the respiration of pellets of mycelium.

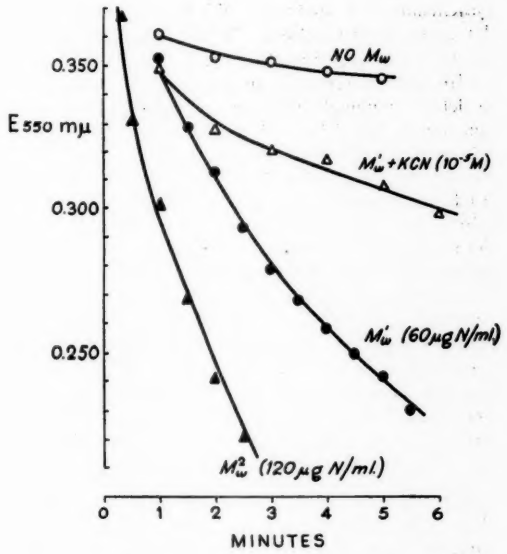


Fig. 4 (*right*). Cytochrome c oxidase activity of isolated particles (M_W). Reaction mixture (μM): reduced cytochrome c, 0.01; phosphate, 75; KCN (where indicated), 0.03; particles (6 or 12 μg nitrogen), and water to 3 ml pH 7.0. Room temperature.

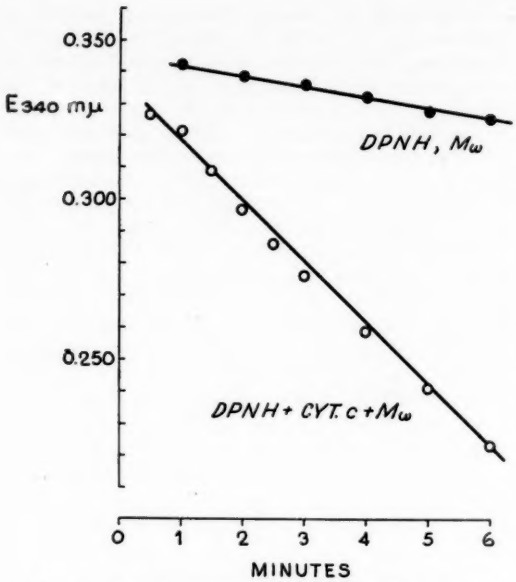


FIG. 5. DPNH oxidase of isolated particles (M_W). Reaction mixture (μM): DPNH, 0.170; cytochrome c (where indicated), 0.01; phosphate, 75; particles (11 μg nitrogen), and water to 3 ml pH 7.0. Room temperature.

the rapid reduction of cytochrome c (fig 6). There was no endogenous reaction and an active DPNH-cytochrome c reductase could be demonstrated; at the same concentration, TPNH was only a tenth as effective an electron donor.

In spite of repeated attempts, it was not possible to detect manometrically any significant oxygen uptake by the particles isolated from hand-ground mycelium using the various tricarboxylic acid cycle intermediates as substrates. However, the particulate fraction obtained after sonic oscillation of the mycelium did consume oxygen at a measurable rate in the presence of succinate. There was no appreciable endogenous respiration, and when succinate was added to the reaction mixture oxygen was taken up at a constant rate (15 μl O_2/hr × mg protein) for at least 20 minutes. Although a wide variety of conditions was tested, it was not possible to demonstrate any esterification of inorganic phosphate during the oxidation of succinate. Under conditions where succinate was oxidized, there was no discernable oxidation of α-ketoglutarate.

SPECTROPHOTOMETRIC OBSERVATIONS ON ISOLATED PARTICLES: The respiratory chain components in particles isolated after sonic oscillation were studied by spectrophotometric techniques. Figure 7 shows a difference spectrum (reduced-oxidized) which compares a suspension of untreated particles with an identical suspension which has been reduced enzymatically by adding DPNH in sufficient excess to exhaust the supply of dissolved oxygen in the cuvette. It is clear that cytochromes of type a (603–605 mμ), b (560–562 mμ), and c (550–552 mμ) are present. The broad band in the 520-530 mμ region represents the fused β-bands of cytochromes b and c. In the Soret region, both the γ-band of cytochrome a-a_3 (443–445 mμ) and the major band of cytochrome b (430 mμ) can be seen; the trough in the 460–480 mμ region indicates the presence of flavoprotein. A rough estimate of the concentrations of the various components can be gained from the difference spectrum, using values for the absorption maxima, reference wave lengths, and extinction coefficients given by Chance and Williams (6). The relative concentrations of the components in the enzymatically-reduced respiratory chain are 1 : 1.4 : 1.6 : 5 for cytochromes a, b, c, and flavoprotein, respectively. It appears that the cytochromes are present in roughly equimolar amounts, whereas flavoprotein is present in molar excess. When the particulate fraction is reduced chemically with sodium hydrosulfite, additional components are reduced (fig 8) and the 445 mμ peak now appears only as a shoulder on the major Soret band.

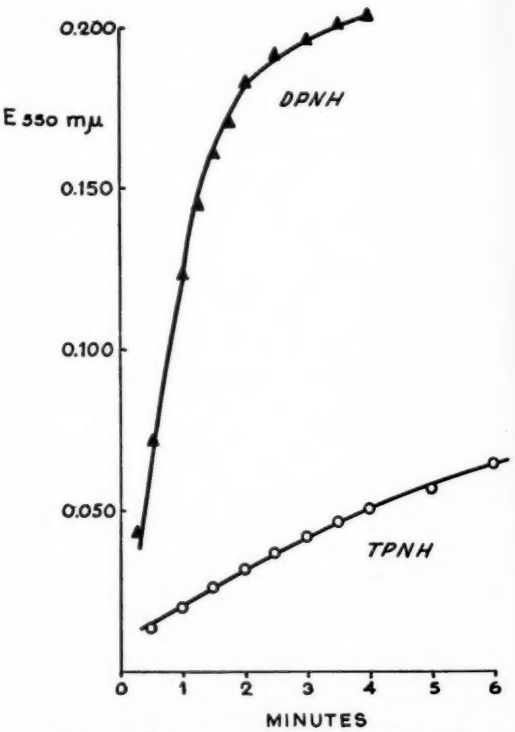
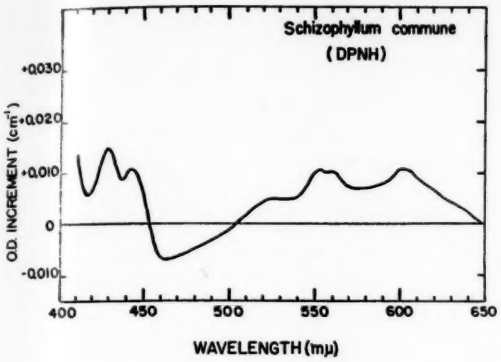


FIG. 6. DPNH- and TPNH-cytochrome c reductase of isolated particles (M_W). Reaction mixture (μM): DPNH or TPNH, 0.120; phosphate, 75; cytochrome c, 0.01; KCN, 0.3; particles (11 μg nitrogen), and water to 3 ml pH 7.0. Room temperature.



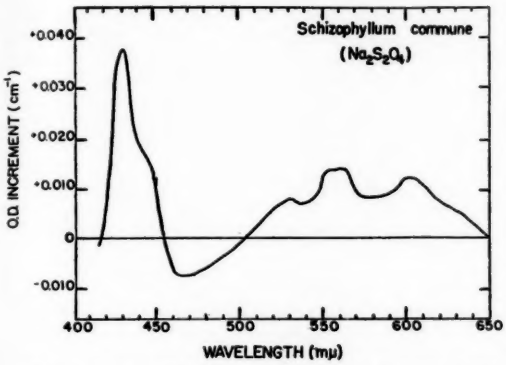


FIG. 7 (*top*). Difference spectrum (reduced with DPNH—oxidized) of isolated particles. Both cuvettes contained 70 μmoles phosphate buffer (pH 7.0), 0.2 ml particles (3 mg protein), and water to 1.5 ml; 4.8 μmoles DPNH added to one. Room temperature.

FIG. 8 (*bottom*). Difference spectrum (reduced with $Na_2S_2O_4$—oxidized) of isolated particles. Both cuvettes contained 70 μmoles phosphate buffer (pH 7.0), 0.2 ml particles (3 mg protein), and water to 1.5 ml; $Na_2S_2O_4$ added to one. Room temperature.

In order to characterize the respiratory pigment which combines with carbon monoxide, the spectral changes caused by flushing an anaerobic suspension of particles with CO were recorded as the (anaerobic + CO)-(anaerobic) difference spectrum. There is a trough at 445 mμ, presumably due to the disappearance of the Soret band of cytochrome a_3, and a broad absorption band between 420 and 430 mμ. The turbidity of the suspensions prevented a more precise definition of the component.

DISCUSSION

The present study has shown that particles isolated from *Schizophyllum commune* contain a typical respiratory chain which can transfer electrons from pyridine nucleotide to molecular oxygen via flavoprotein and the cytochrome system. The effects of respiratory inhibitors on the mycelium lend strong support to the view that this chain is functional in the intact tissue. By blocking electron transfer between cytochromes b and c with antimycin it is possible to eliminate virtually all of the respiration. The nature of the terminal oxidase is well established by demonstrations that A: Mycelium respiration is light-reversibly inhibited by CO and B: The isolated particles show cytochrome c oxidase activity and contain a component which is spectrally similar to cytochrome a_3. Although the rate of respiration declines markedly as the mycelium ages, there is no evidence for any qualitative change in the electron transfer system.

The type of respiratory chain described here is apparently very general among the fungi. Fungal respiration is characteristically cyanide-sensitive (1, 14, 18, 23, 26), although some interesting cases of cyanide- and CO-insensitivity have been reported (9). The respiration of the basidiomycetes *Polystictus versicolor* (2) and *Puccinia graminis* tritici (25) is inhibited by CO and this effect is reversed by light, indicating that here too the electron transfer is mediated by cytochrome oxidase. A spectroscopic survey of 45 species of fungi, including 13 basidiomycetes, showed that a complete cytochrome system was present in every case (3). Cytochrome oxidase activity has been reported in extracts of a variety of fungi (5, 11, 13, 19, 20), and cytochrome c has been purified from the basidiomycete, *Ustilago sphaerogena* (15). The electron transfer system is presumably localized on the mitochondria. No attempt was made in the present study to define the morphology of the *S. commune* particles, but a typical mitochondrial fine structure has been shown in a related basidiomycete, *Coprinus disseminatus* (10).

There is some evidence for the general role of the tricarboxylic acid cycle in the respiration of the fungi (7). Although the *S. commune* particles did oxidize succinate, it was not possible to demonstrate the activity of the entire cycle. After completing this work, the results of Wessels (24), demonstrating the operation of a complete tricarboxylic acid cycle in *S. commune* particles, came to our attention. His isolation procedure differed from those used here in the inclusion of 0.8 M glucose and nicotinamide in the medium; these conditions probably maintain the mitochondria in a more physiological state. Wessels was also able to demonstrate the oxidation of exogenous organic acids by the dried mycelium. The relatively high endogenous respiration of the intact mycelium, and its response to exogenous glucose, may be related to the fact that 10-day-old tissue was used, in contrast to the 3-day-old mycelium employed here.

SUMMARY

The respiratory characteristics of *Schizophyllum commune* mycelium grown on solid and liquid media

have been examined. The oxygen uptake can be markedly inhibited by cyanide, azide, CO (in the dark only), antimycin A, and phenylmercuric acetate, while copper-combining agents have little effect. At low concentrations, DNP stimulates the respiration and inhibits growth, but at higher concentrations both processes are inhibited.

Particles (mitochondria) isolated by differential centrifugation from homogenates of the mycelium showed the following activities: cytochrome c oxidase; DPNH-, TPNH-, and succinic-cytochrome c reductases; DPNH oxidase; succinoxidase. Spectrophotometric observations of the particle components which are reduced by DPNH revealed the presence of roughly equimolar concentrations of a-, b-, and c-type cytochromes and a molar excess of flavoprotein. It is concluded that respiration in *S. commune* mycelium involves the transfer of electrons from substrates to molecular oxygen through a chain which includes these components.

Acknowledgments

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UBIQUITY OF STIMULATION OF ENDOGENOUS RESPIRATION BY PURINES & PURINE ANALOGS[1]

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In a recent publication (2) we reported a striking stimulation of the endogenous respiration of nitrogen starved *Chlorella vulgaris* (Beijerinck) by relatively low concentrations of a large number of purines and purine analogs. Since this observation pointed to a new regulatory function of the purines in Chlorella metabolism, it was of interest to look for a similar effect in other tissues and organisms. This paper reports the stimulation of the respiration of a large variety of nitrogen-starved tissues by purines and purine analogs.

METHODS & MATERIALS

Cultures of algae were obtained from the American Type Culture Collection (ATCC) or the Culture Collection of Algae at Indiana University (CCAIU) as indicated in each case. Yeast cultures were a gift of Dr. S. S. Jackel, Fleischmann Laboratories, Standard Brands, Inc. The culture of *Parthenocissus tricuspidata* (Sieb. & Zucc.) Planch. crown gall (Morel) was obtained from Dr. Jaques Lipetz, Botany Department, Yale University. Seeds of higher plants were purchased locally.

Anacystis was cultured on medium C of Kratz and Myers (7). Euglena was cultured as described (1). The other algae were cultured in a modified Bristol's medium (3) plus Hoagland's A–Z microelements solution, 0.1 ml/l (5), iron versenate, 0.3 ml/l (6), 1% polypeptone (Baltimore Biological Lab.), and 0.1 ml/l Dow Corning Antifoam B, at pH 6.0 in 7 liter bottles (1). The yeast cultures were grown with agitation in 1 liter Erlenmeyer flask in the SAS base of Schultz and Atkin (8) supplemented with 5% glucose, and 3% trypticase soy broth (Baltimore Biological Lab.), pH 5.2. The cultures were harvested aseptically by centrifugation. For nitrogen starvation the organisms were resuspended in the original volume of a new medium, which differed from the original medium only in that all nitrogen sources (including polypeptone, etc.) had been omitted. The yeast medium for nitrogen starvation was supplemented with 15% glucose.

The Parthenocissus crown gall tissue was grown in liquid suspension with vigorous aeration in Klein's modification of White's medium (6) in 7 liter bottles. This medium was modified further by replacing 90% of the $Ca(NO_3)_2$ with KNO_3. The tissue was harvested and nitrogen starved in the same manner as the algal cultures. Before use the cell clumps were briefly homogenized in a loose-fitting conical glass homogenizer to allow more uniform pipetting. Microscopic examination showed little cell breakage by this procedure.

Peanuts and castor bean seeds were washed and surface sterilized by brief immersion in dilute sodium hypochlorite solution. They were germinated under aseptic conditions on sterilized wet filter paper in petri plates. One week after germination (4) the cotyledons were harvested and peeled. They were then incubated with agitation in sterile distilled water for 1 to 2 weeks. These leached cotyledons were harvested and the discolored cortical tissue was discarded. The remaining tissue was washed in distilled water, dried briefly on paper towels, sliced and randomized. Weighed aliquots were distributed into Warburg flasks containing 2.5 ml 0.067 M phosphate buffer, pH 6.0. Respiration was expressed as μliter/O_2/mg fresh weight. All other methods used have been described previously (2).

RESULTS & DISCUSSION

Tables I and II summarize the qualitative results which were obtained with the various tissues studied. The numerical ratings of the various compounds are approximate relative orders of activity, four being the most active and zero being inactive. As can be seen responses varied widely with regard to specificity for the various compounds tested. It is of interest that all the tissues which showed response to purines are also known to contain or accumulate large fat reserves (9). In no instance could a response to purines be demonstrated in tissues which were not nitrogen starved or leached. The absence of a demonstrable response to purines in the peanut cotyledons is possibly due to insufficient nitrogen starvation. This is supported by the observation that, while the aerated castor bean tissue had essentially no endogenous respiration and a substrate had to be added before the effect of purines could be observed, peanut cotyledons retained a considerable endogenous respiration even after 2 weeks of aeration in distilled water. Slicing the peanut cotyledons before the

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TABLE I

EFFECT OF VARIOUS PURINES & PURINE ANALOGS ON RESPIRATION OF NITROGEN-STARVED TISSUES*

ORGANISM	N STARVATION PERIOD DAYS	8-AZAGUANINE	6-MERCAPTOPURINE	HYPOXANTHINE	ADENINE	URIC ACID	6-CHLOROPURINE	6-CYANOPURINE	2-AZAHYPOXANTHINE	PURINE
Algae										
Anacystis nidulans (CCAIU 625)	7–21	0	0	...	0	0	...	...	...	...
Ankistrodemus falcatus (CCAIU 188)	7–14	4	3	2	1	1	0	...	0	...
Chlorella ellipsoidea (CCAIU 20)	7–14	0	1	3	2	...	...	...	...	...
C. luteoviridis var. aureoviridis (CCAIU 22)	7–14	3	2	2	4	...	...	...	...	...
C. pyrenoidosa (ATCC 11469)	5	3	...	...	4	...	...	2	3	4
C. variegata (CCAIU 28)	14	3	...	...	4	1	2	...	0	1
C. vulgaris (ATCC 11468)	5	3	4	2	3	2	2	3	4	4
C. vulgaris (CCAIU 259)	5	3	...	...	4	...	...	3	2	2
C. vulgaris (CCAIU 29)	7	...	...	...	4	...	...	1	...	3
C. vulgaris var. viridis (CCAIU 396)	6	3	...	...	2	...	...	4	3	2
C. xanthella (CCAIU 31)	14–21	4	0	3	4	2	0	2	...	...
Chloridella minuta (CCAIU 491)	14–21	2	3	0	4	...	...	...	...	...
Chlorococcum macrostigmatum (CCAIU 109)	14–21	4	3	1	2	...	...	...	...	...
Coelastrum microporum (CCAIU 280)	7–10	4	0	...	4	2	1	2	1	...
Euglena gracilis st. Z (CCAIU 753)	7	0	0	0	2	...	...	...	...	...
Yeasts										
*Saccharomyces cerevisiae***	7–21	0	0	...	0	0	...	...	...	...
Torula utilis	14	3	1	...	0	4	...	...	...	...
Higher plants										
Arachis hypogaea (L.) cotyledons	14	0	0	...	0	0	...	...	...	...
Parthenocissus tricuspidata crown gall (Morel)**	5	...	...	...	2	...	...	...	...	...
Ricinus communis (L.) cotyledons**	7	2	0	...	0	0	...	...	...	...

* Stimulation of endogenous respiration is recorded four being maximal (approximately 3-fold stimulation) and 0 indicating no effect. A response rated as one could well be due to oxidation of the purines.

** 5×10^{-3} M glucose or 5×10^{-3} M linoleic acid was added as substrate since endogenous respiration ± purines was nil.

aeration treatment did not improve this situation. Thus, with the exception of the peanuts, all the tissues in which no response to purines could be demonstrated were tissues which are not usually considered to accumulate fats.

The magnitude of the response to purines varied widely among the tissues. Thus in some instances stimulations of only 30 % above endogenous respiration were achieved, while in other cases stimulations of 300 % were observed. Nonetheless, an examination of the degree of oxidation of the purines, as measured by liberation of $C^{14}O_2$ from C^{14}-labeled 8-azaguanine, and a comparison of the stimulations obtained with the purines to those obtained with equimolar amounts of inorganic nitrogen compounds clearly exclude the possibility that the stimulations can be explained as nitrogen effects or by the oxidation of the purines. Table III summarizes these results. It will be noted that in many instances no information was available on the fraction of the added purine which had been oxidized by the cells. In these cases the added purines were assumed to have been completely oxidized and the ratios were calculated on this basis. This is obviously an extreme assumption since when the experiments were terminated at the end of 3 to 6 hours respiration was still continuing at a constant and undiminished rate. Furthermore, comparison to those tissues where C^{14} data are available clearly show that the actual rate of oxidation of these compounds is very low in all cases. In a similar manner, the calculations involving nitrogen release from the purines have assumed the presence of a guanase in all cases and the complete liberation of the amino group of 8-azaguanine as ammonia. These assumptions necessarily tend to make the results in the last column minimum values.

Inhibitor studies and other biochemical experiments with *Chlorella vulgaris*, which will be reported in detail elsewhere, suggest that the effect of purines in stimulating respiration is connected to the fat metabolism of this organism. As far as these results have been checked with some of the tissues discussed here, the results have been strictly comparable. Our ability to predict with fair accuracy which tissues are likely to show a response to purines after nitrogen starvation, merely on the basis of their reported tendency to store fat under these conditions, encourages the belief that these observations may help shed some light on the metabolic changes which accompany nitrogen starvation and fat accumulation in general.

Summary

Results have been presented which show that various purines and purine analogs stimulate the respiration of a large variety of nitrogen-starved tissues and organisms which are known to store fat during nitrogen starvation. The respiration caused by the purines is a stimulation of the respiration of endogenous substrates in the cells and cannot be explained either by oxidation of the purines or by stimulation which could be expected from the ammonia released during their oxidation.

Acknowledgments

The able technical assistance of Miss Roberta J. Thomas and Miss Barbara A. Edwards is gratefully acknowledged.

Table II

Effect of Additional Purines & Purine Analogs on Respiration of Nitrogen-Starved Tissues

	N Starvation Period Days	1,3-Dimethylxanthine	Imidazo-(b)-pyrazine	6-*n*-Butylaminopurine	Guanine	4-Aminopyrazolopyrimidine	4-Mercaptopyrazolopyrimidine	Xanthine	Kinetin	1-Methyladenine	8-Azadenine
Chlorella vulgaris (ATCC 11469)	5	1	0	0	...	...	...	...	...	...	...
C. variegata (CCAIU 28)	14	...	...	0	...	...	0	...	0	0	...
C. vulgaris (ATCC 11468)	5	1	1	0	2	2	0	2	1	3	...
C. vulgaris (CCAIU 259)	5	1	1	1	...	...	...	...	...	...	...
C. vulgaris (CCAIU 29)	7	...	...	0	...	...	...	...	...	...	...
C. vulgaris var. viridis (CCAIU 396)	6	0	0	1	...	...	...	0	...	...	...
C. xanthella (CCAIU 31)	14–21	...	...	...	1	...	...	...	...	...	0
Coelastrum microporum (CCAIU 280)	7–10	...	0	...	...	0	...	...	...	...	3

TABLE III

QUANTITATIVE ASPECTS OF STIMULATION OF RESPIRATION BY PURINES

ORGANISM	CONC FOR OPTIMAL ACTIVITY		MAX. STIMUL. (% ENDOG.)	% PURINE OXIDIZED	EXCESS O_2 UPTAKE*	STIMULATION BY EQUIV. $(NH_4)_2SO_4$ CONTENT**	MAX. STIM. BY $(NH_4)_2SO_4$ $\mu M\ O_2/\mu M\ NH_4^+$	MAX. STIM. BY PURINES $\mu M\ O_2/\mu M\ N$ RELEASED***
	COMP'D.	CONC M						
Ankistrodemus falcatus	Azg†	4×10^{-4}	200	0.34	500	110	0.86	100††
Chlorella ellipsoidea	Hx	3×10^{-4}	180	...	1.3‡	110	0.78	1.6‡
C. luteoviridis var. aureoviridis	Adn	2×10^{-4}	210	...	1.3‡	...	0.36	1.3‡
C. pyrenoidosa	Pur.	10^{-4}	220	...	3.6‡	...	1.6	4.5‡
C. variegata	Adn	3×10^{-4}	230	...	1.6‡	...	0.80	1.6‡
C. vulgaris (ATCC 11468)	SHP	10^{-4}	290	0.16	6150	...	3.2	77,000
C. vulgaris (CCAIU 259)	CNP	10^{-4}	190	...	5.7‡	...	1.0	7.2‡
C. vulgaris (CCAIU 29)	Pur.	10^{-4}	200	...	1.3‡	...	0.43	1.6‡
C. vulgaris var. viridis (CCAIU 396)	CNP	10^{-4}	260	...	7.7‡	...	0.83	9.6‡
C. xanthella	Azg	10^{-4}	240	2.1	330	160	0.22	25††
Chloridella minuta	Azg	10^{-4}	130	...	1.9‡	140	0.92	1.3‡
Chlorococcum macrostigmatum	Azg	10^{-4}	300	...	8.6‡	100	1.4	5.7‡
Coelastrum microporum	Azg	5×10^{-4}	252	0.42	255	155	1.1	4.2††
Torula utilis	Azg	3×10^{-5}	174	0.60	1600	100	0.36	36††

* Excess oxygen uptake $=$ $\dfrac{\mu l\ O_2 \text{ uptake in presence of purines} - \mu l \text{ endogenous } O_2 \text{ uptake}}{\mu l\ O_2 \text{ required for complete oxidation}}$

** % Stimulation by equivalent $(NH_4)_2SO_4$ conc $=$ $\dfrac{\mu l\ O_2 \text{ uptake in presence of } (NH_4)_2SO_4}{\mu l \text{ endogenous } O_2 \text{ uptake}} \times 100$; The equivalent $(NH_4)_2SO_4$ concentration is that concentration which equals the total nitrogen content of the added purine.

*** These numbers are minimum values since respiration was linear with time and the rate had not changed when the experiments were terminated.

† The following abbreviations are used: Azg = 8-azaguanine, Hx = hypoxanthine, Adn = Adenine, Pur. = purine, SHP = 6-mercaptopurine, CNP = 6-cyanopurine.

†† The μM N released are calculated on the basis of the observed % total oxidation, assuming 5 μM N released/μM azaguanine oxidized. In addition all the nitrogen from the 2-NH_2 group is assumed to be released since azaguanine is rapidly converted to azaxanthine.

‡ Since no information is available on the degree of oxidation, the results are computed on the assumption of total oxidation of the added purines.

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CORRELATION BETWEEN INACTIVATION OF 2,4-DICHLOROPHENOXYACETIC ACID & CESSATION OF CALLUS GROWTH IN BEAN STEM SECTIONS[1]

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In a previous publication (1) we have described an in vitro system for studying the callus growth on bean stem sections which is initiated by applying relatively high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). Only short exposure to 2,4-D was necessary to initiate the callus growth. Since this growth is not tumorous in nature and therefore not auxin autonomous, it will continue only as long as there is a supply of the growth inducing material still present in the tissue. It was of interest, therefore, to follow the fate of C^{14}-labeled 2,4-D in this system and to attempt to correlate it with the data on callus growth. This paper will report the results of these experiments.

METHODS & MATERIALS

The methods for harvesting bean stems and growing them aseptically on nutrient agar were the same as previously described, except that the amount of agar in the petri plates was kept constant, so as to control the depth to which the stems were implanted. In all cases exposure to 2,4-D was for 24 hours except in the CO_2 trapping experiments, where the 2,4-D blocks were kept in place for the full duration of the experiment. Because the amount of radioactivity which was secreted into the medium from the basal ends of the stems was very low, it was necessary to concentrate large amounts of medium to obtain accurate estimates of the amount of radioactivity present and to determine the nature of the radioactive compounds involved. Since the agar medium did not lend itself to concentration and subsequent paper chromatography, a method for obtaining proliferations in liquid medium was developed. For this purpose, specially constructed stainless steel holders were used to maintain the stem sections in a vertical position with their lower ends in the liquid medium. The agar blocks were placed on top of the stems while the stems and holders were placed in an empty, sterile petri plate to avoid contaminating the medium with radioactive 2,4-D should the agar blocks drop off in the course of the manipulations.

The assembled units (holders, stems, & 2,4-D blocks) were then transferred to new sterile petri plates or other containers and the medium added. When the evolution of $C^{14}O_2$ from C^{14}-labeled 2,4-D was studied, the holders were placed in 100 ml beakers which had been cut off to a height of 25 mm. Sixteen stems were placed in each beaker along with 10 ml of medium. The beakers were closed with rubber stoppers which had been cut to a thickness of 10 mm and sealed with a beeswax-rosin mixture. The rubber stoppers had two glass tubes leading through them. One tube was connected to a source of sterile, humidified, CO_2-free air, and the other tube to a bubbler in a 12 ml centrifuge tube for trapping the evolved CO_2 in 15% KOH solution. Eight such beakers were arranged in parallel and shared the same source of treated air. The flow of air through each beaker was regulated to keep the bubbling rate in the CO_2 traps constant. The recovery of respired CO_2 was checked with yeast cultures respiring C^{14}-labeled glucose in this system. Recoveries of 95% were obtained routinely.

At the end of the experiment, the trapped CO_2 was precipitated by adding a few drops of 12% $BaCl_2$. A check for completeness of precipitation was made after the precipitated $BaCO_3$ was removed by centrifugation. The agar blocks for each vessel were collected and pooled. They were melted by heating with a few drops of water, adjusted to volume, and aliquots counted. The medium was concentrated under vacuum and the stems were extracted with alcohol. Aliquots of the extract, the fibrous residue, and the concentrated medium were counted. Separate aliquots of the stem extracts and the concentrated medium were also spotted on chromatograms and developed as described (1).

RESULTS & DISCUSSION

Figure 1 shows the percent increase in fresh weight of bean stem sections which received one and two consecutive applications of 2,4-D. Each point represents the average of five replicates, each of which

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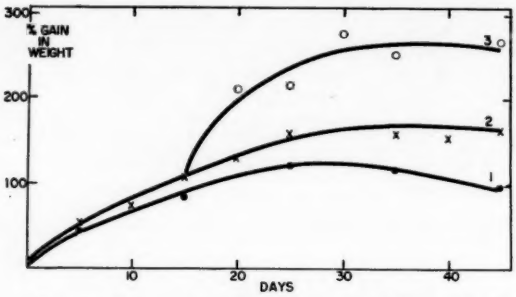

FIG. 1. Effect of reapplication of 2,4-D on fresh weight of bean stem sections. Curve 1, control. Curve 2, 10^{-4} M 2,4-D agar blocks applied for 24 hours at beginning of experiment. Curve 3, 10^{-4} M 2,4-D agar blocks applied for 24 hours at beginning of experiment and again after 15 days.

consisted of five stem sections. Controls were treated identically to exposed stems except agar blocks free of 2,4-D were applied. It will be noted that a second application of 2,4-D, after growth from the first application had slowed down, resulted in a new spurt of growth, equal in magnitude to the first. It would appear, therefore, that the cessation of growth in this system was not due to the exhaustion of any nutrients in the medium but rather to the exhaustion of the growth inducer, 2,4-D.

As mentioned previously (1) it was not possible to obtain proliferation in bean stems floated on a liquid medium. Further investigation indicated that the proliferation process was strongly aerobic and that the floated sections apparently did not receive enough oxygen. Similarly, when stems were supported in the specially designed holders in closed vessels, no growth was observed in the absence of circulating air, even when CO_2 was absorbed by KOH in center wells. If, however, the beakers were continuously aerated, growth comparable to that observed on agar was obtained.

Table I summarizes the fate of the radioactivity from C^{14}-carboxyl labeled 2,4-D in the course of a growth experiment on liquid medium. Each value is the average of four individual determinations, using 16 bean stems in each determination. Note that the amount of radioactivity liberated as $C^{14}O_2$ was quite low, and seemed to increase only toward the end of the experiment. In spite of all efforts to maintain aseptic conditions, it did not prove feasible to completely prevent contamination of the medium. It is very possible, therefore, that the apparent increase in $C^{14}O_2$ liberation toward the end of the experiment is actually due to bacterial oxidation of the radioactive metabolites of 2,4-D which were slowly released into the medium. The apparent failure of the radioactivity in the medium to increase in the latter part of the experiment also agrees with this explanation. Most of the radioactivity which had been taken up by the stems was in an ethanol-soluble form in the stems at the end of the experiment. Paper chromatographic analysis of these extracts revealed that the radioactivity was almost exclusively in the band at R_f 0.5 in Butanol-propionic acid-H_2O (8:5.6:12 v/v/v) (3). The radioactivity in the medium, on the other hand, moved near the front in this solvent, as did 2,4-D. There was only little if any binding of radioactivity to the ethanol-insoluble residue of

TABLE I

BALANCE SHEET OF FATE OF C^{14}-CARBOXYL LABELED 2,4-D IN BEAN STEM SECTIONS

FRACTION	% OF TOTAL APPLIED RADIOACTIVITY		
	1ST DAY	3RD DAY	7TH DAY
Agar blocks	53±5	24±0.4	23±0.4
Bean extracts:			
R_f 0.5 Complex	16.6±1.5	42.8±1.8	46.5±1.8
Free 2,4-D	27.4±2.5	19.2±0.8	11.5±0.4
Medium	1.63±0.06	5.5±0.6	5.7±0.2
Respired CO_2	0.76±.02	3.5±0.02	8.3±0.3
Fiber residue	1.51±0.09	4.4±0.4	4.6±0.3
Total recovery	106±6.3	101±4.3	97.2±4.0

TABLE II

CORRELATION BETWEEN GROWTH & INACTIVATION OF APPLIED C^{14} LABELED 2,4-D

TIME DAYS	% GAIN IN FRESH WT		ETHANOL EXTRACT CPM/TOTAL SAMPLE	DISTRIBUTION OF RADIOACTIVITY (%)	
	2,4-D TREATED	CONTROL		R_f 0.5 COMPLEX	2,4-D
2	44±3	3.2±0.9	67.4±2.1	44	55
4	71±4	27±3	74.6±6.0	72	28
7	142±35	29±18	64.4±3.3	87	13
8	95±11	23±3	69.9±3.7	87	13
10	144±16	67±6	58.0±3.5	89	10
15	176±17	58±14	60.7±5.6	88	12
21	188±21	106±4	62.4±5.2	97	3

the cells. These results are largely in agreement with those obtained by Weintraub et al (4) with whole bean plants.

The radioactivity from 2,4-D remained in the stems for very considerable lengths of time (table II). In this experiment the growth [weight gain expressed as (final wt-initial wt) × 100/initial wt] of the stems was measured along with the radioactivity which remained in them in ethanol-extractable form. Paper chromatography of the extracts revealed that the compounds moving at R_f 0.5 were essentially the only detectable source of radioactivity after the 15th day of incubation. Thus there appears to be a good correlation between the disappearance of detectable amounts of free 2,4-D and the cessation of growth. Apparently the compounds moving at R_f 0.5 can be regarded as true detoxification products of 2,4-D in that they remain in the tissue but appear to exert no effect on it. Hay and Thimann also have implied the presence of such a detoxification product in bean seedlings (2). It will be of interest to verify this point by direct experimentation. For this purpose purified preparations of the compounds of R_f 0.5 are required since crude isolates of this material appeared to be quite toxic to the tissues and inhibited all growth in bioassays. The purification of this material and studies of its chemistry and biological effects will constitute the subject of further publications.

Summary

Results have been presented which correlate the cessation of growth of excised bean stem sections treated with 2,4-D, with the disappearance of free 2,4-D from the sections. The radioactivity from C^{14}-carboxyl labeled 2,4-D largely remained in the tissue in an ethanol-soluble form chromatographically distinct from 2,4-D and only relatively small amounts of radioactivity were found in CO_2 or released into the medium.

Acknowledgments

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FURTHER STUDIES OF SYNERGISM IN SLIT PEA TEST[1,2]

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The work reported in this paper, which is a continuation of that presented earlier (9), had two primary objectives. The first was to determine whether or not sparing of IAA[3] by IAN from IAA oxidase could be involved in IAN-IAA synergism in the slit pea test. The second was to determine more completely the kind and extent of growth occurring.

If IAA oxidase were of major importance in the interaction of IAN-IAA, no synergism should be expected with either NAA or 2,4-D, which presumably are not attacked by the oxidase (5, 13). Van Raalte (18) found that in root production indole synergized with IAA but not with NAA. He considered this suggestive that sparing of IAA from IAA oxidase was involved and presented evidence that at high concentrations of IAA indole inhibits the oxidase. The physiological significance of the latter already has been questioned (9) on the basis that at the lowest concentration of IAA tested, 6×10^{-4} M and still above physiological, indole seemingly stimulated the oxidase.

If either or both NAA and 2,4-D interact synergistically with IAN, IAA oxidase can be eliminated from further consideration in attempts to explain IAN-IAA synergism in the slit pea test. Comparable responses to NAA and IAA have been used by Hillman (5) to reinforce the argument that changed auxin sensitivity of red-grown pea internodes could not be ascribed to changes in IAA oxidase activity.

Compounds other than IAN and indole have been reported to synergize with IAA. It seemed advisable, also, to investigate whether or not IAA oxidase could be involved in any of these instances. Besides IAN and indole, the following were checked: NAN; the hemiauxins, CHAA and PBA, of Went (19); and skatole, which Thimann (14) reported to have some synergistic activity with IAA in the pea curvature test.

The second objective was accomplished by measuring and comparing changes in curvature, average length, fresh weight, cross-sectional area, and cross-sectional shape. Both whole and slit sections were used.

MATERIALS & METHODS

Seeds of *Pisum sativum* L. var. Alaska were soaked 6 hours and planted in vermiculite in polystyrene trays. The etiolated plants were grown in a dark room. At irregular intervals, whenever it was necessary to be in the dark room, seedlings were exposed to dim red light from incandescent ruby safe lamps (NALCO, 8 C.P.).

In an attempt to find a possible reason for variations in sensitivity of the slit test from one experiment to another that had been mentioned in the literature, Kent and Gortner (6) investigated the effects on pea seedlings of amount and time of exposure to red light. Although a great influence was demonstrated, unfortunately, the standard errors reported and the small amount of replicate data presented indicated considerable variability among their sections both within and between experiments. With the irregular exposures to red light used, this author has generally experienced adequate sensitivity and better replicability than Kent and Gortner; therefore it was felt that the inconveniences involved and the accompanying heat radiated from incandescent lamps of the type used by them made it unnecessary and impractical to attempt a rigorous standardization of light exposure in this or in previous work (9).

Temperature was controlled to 24±2° C. Malfunctioning equipment allowed the temperature to rise, on one occasion, to 32° C for 8 hours and, on a second, to gradually fall to 17° C during 36 hours. Both occurred during the 2nd to 3rd day of growth and neither appeared to influence the responses of sections taken, as usual, 7 days after planting.

Relative humidity fluctuated between 50 and 90 %.

Where applicable, techniques and apparatus were as previously described (8, 9). All sections were cut starting 5 mm below the hook near the apex. Only third internodes between 40 and 55 mm long were used. Sections for the slit test were initially 35 mm long unless otherwise noted.

Because fresh weight of individual sections may be more readily and accurately determined than volumes and because volume increase is primarly dependent on water uptake, changes in fresh weight were used as measures of volume changes. Fresh weight values also were used in calculation of cross-sectional areas. This seemed justifiable because the specific gravity of pea sections is close to one and seems to change little during the types of experiments reported here and because calculations using the data of Galston, Baker, and King (2) show weight and volume changes over a wide range of magnitude to be nearly identical.

Sections to be weighed were cut to be initially 10 or 20 mm long. Slit 10 mm sections were slit their entire length into two halves. Slit 20 mm sections

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[2] Supported in part by National Science Foundation Research Grant G9015.

[3] The following abbreviations are used: CHAA, cyclohexaneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, 3-indoleacetic acid; IAN, 3-indoleacetonitrile; NAA, 1-naphthaleneacetic acid; NAN, 1-naphthaleneacetonitrile; and PBA, γ-phenyl-*n*-butyric acid.

were slit for 17 mm. One to two hours after being cut and floated on water, sections were blotted with filter paper and rapidly weighed on a 125 mg capacity Roller-Smith Precision Balance. Records of initial curvatures and/or lengths were obtained immediately following weighing either by shadowgraphing or by direct measurement. After 24 hours in test solutions, sections were again blotted, weighed, shadowgraphed, and/or directly measured. Lengths of shadowgraphed sections were obtained by use of a map measure directly on images projected from an opaque projector. Average lengths of curved sections were obtained by guiding the map measure along the estimated midlines of the images.

Photomicrographs were made of cross sections of fresh material cut freehand using a razor blade. The sections were taken from the regions of maximum curvature, unstained, and mounted in water.

The term, curvature, always refers to the absolute curvature and was measured by the stem reference method (9, 15), in which the angle measured is between a line tangent to the curvature at the tip of an arm and the line longitudinally bisecting the unslit basal portion of the stem. In 10 mm sections with no unslit basal portion, the angle between lines tangent to each tip was measured. Curvature difference, unless otherwise specified, refers to the difference in curvature between sections in test solutions and sections in water. Curvature change refers to the change of curvature that occurs in one sample between the times of placing in and removing from test solution.

IAN, NAN, CHAA, PBA, indole, and skatole, which have little or no activity when used alone in the pea curvature test, will be referred to as additives.

In some experiments sections were pooled so that individual changes were unknown and could not be analyzed statistically. In other experiments the sections were separated in compartments cut into polyethylene disks (7) and were followed individually. These and other data were analyzed and are given with standard errors of means. Sample size was 10 or 20 and is indicated in each table.

Results

The curvature and straight growth responses reported in this paper and obtained in Athens have been consistently lower than those previously observed in Iowa City (9). No obvious reason has been found; and, since essentially the same procedures were followed and comparable experiments produced the same relative results, e.g., considerable IAN-IAA synergism in curvature at 10^{-6} M IAA but none at 10^{-5} M IAA, they are presented without qualification.

Tables I, II, and III present the results of experiments pursuant to objective one. In each of these tables the column headed "synergism" is of most interest. To calculate the amount of synergism, if any,

Table I

Effects of Various Additives on Slit Pea Curvatures in IAA*

Additive	M Conc	Curvature in additive	Curvature in water	M Conc IAA	Curvature in IAA	Curvature in IAA + additive	Synergism**
IAN	3.2×10^{-4}	...***	−126±7	1×10^{-6}	−85±2	20±8	105
	3.2×10^{-4}	...***	−144±5	1×10^{-5}	51±13	50±12	−1
	1×10^{-3}	−68±7	−126±7	1×10^{-6}	−85±2	16±17	43
NAN	3.2×10^{-5}	−145±7	−142±8	1×10^{-6}	−66±6	187±10	253
	1×10^{-4}	−120±7	−142±8	1×10^{-6}	−66±6	158±8	224
	3.2×10^{-4}	−232±13	−148±13	1×10^{-6}	−92±6	−56±28	120
CHAA	1×10^{-4}	−142±12	−162±10	1×10^{-6}	−88±15	90±19	178
	3.2×10^{-4}	−137±12	−162±10	1×10^{-6}	−88±15	44±12	132
PBA	1×10^{-5}	−150±12	−162±10	1×10^{-6}	−88±15	−55±13	33
	1×10^{-4}	−64±10	−162±10	1×10^{-6}	−88±15	80±24	70
	1×10^{-3}	−182±24	−162±10	1×10^{-6}	−88±15	−70±17	18
Indole	1×10^{-4}	−134±11	−142±8	1×10^{-6}	−66±6	−74±8	−8
	3.2×10^{-4}	−161±8	−142±8	1×10^{-6}	−66±6	−50±6	16
	1×10^{-3}	−164±14	−142±8	1×10^{-6}	−66±6	−39±14	27
Skatole	1×10^{-5}	−160±10	−148±13	1×10^{-6}	−92±6	−104±10	−12
	1×10^{-4}	−143±13	−148±13	1×10^{-6}	−92±6	−79±14	13
	1×10^{-3}	−132±16	−148±13	1×10^{-6}	−92±6	−78±10	14
	3.2×10^{-4}	−156±10	−141±9	1×10^{-5}	133±17	130±15	−3

* In most samples $n = 10$; in a few, $n = 20$.

** Difference between curvatures in IAA and IAA + additive unless additive, alone, had significant effect. If so, corrected for this effect.

*** Other experiments gave values insignificantly different from curvature in water.

TABLE II

EFFECTS OF VARIOUS ADDITIVES ON SLIT PEA CURVATURES IN NAA*

ADDITIVE	M CONC	CURVATURE IN ADDITIVE	CURVATURE IN WATER	M CONC NAA	CURVATURE IN NAA	CURVATURE IN NAA + ADDITIVE	SYNERGISM**
IAN	3.2×10^{-4}	−134±11	−141±9	1×10^{-6}	−82±8	−8±16	74
	3.2×10^{-4}	−134±11	−141±9	2×10^{-6}	34±13	86±17	52
	3.2×10^{-4}	...***	−144±5	1×10^{-5}	62±10	24±11	−38
	1×10^{-3}	−68±7	−126±7	1×10^{-6}	−97±7	−10±9	29
NAN	3.2×10^{-5}	−114±6	−141±9	1×10^{-6}	−82±8	83±20	165
	3.2×10^{-5}	−114±6	−141±9	2×10^{-6}	34±13	192±19	158
CHAA	3.2×10^{-4}	−137±12	−162±10	1×10^{-6}	−94±10	−12±15	82
PBA	1×10^{-5}	−131±9	−141±9	1×10^{-6}	−82±8	−43±17	39
	1×10^{-5}	−131±9	−141±9	2×10^{-6}	34±13	60±9	26

*, **, and *** As in table I.

it is necessary to know the curvatures obtained in water, additive, auxin, and additive plus auxin; therefore these values are also presented for reference.

IAN, NAN, and CHAA showed significant synergism with both IAA and NAA, though that with NAA was somewhat less throughout than that with IAA. NAN was a more effective synergist with both IAA and NAA than either IAN or CHAA, the latter showing about equal to slightly greater effectiveness than IAN. PBA showed synergistic tendencies that were not significant.

Indole did not interact synergistically with IAA in the slit pea test, although it did in root production (3, 18). Skatole also did not show synergistic activity. The latter is at variance with the data of Thimann (14).

Because of the lack of interaction between either indole or skatole and IAA, these were not used with NAA or 2,4-D.

None of the additives synergized with 2,4-D, in fact, they frequently reduced the curvature that would have been obtained by 2,4-D alone.

Although it was not the objective of these experiments to determine the effects of additives used alone, it was necessary to do so to evaluate properly possible synergistic interactions with the auxins. Several points of interest may be noted in the results obtained.

At a concentration of 10^{-3} M, IAN showed activity approximately equal to 10^{-6} M IAA. In many experiments the author had found (9) and has continued to find 3.2×10^{-4} M IAN inactive in 35 mm slit sections. This concentration did show slight activity (significant at the 5 % level) with 20 mm slit sections (table IV); and it was reported by Osborne (10) to have activity in the slit pea test as performed by her. Apparently IAN has some activity at high enough concentration.

Although NAN seemed to react much like IAN

TABLE III

EFFECTS OF VARIOUS ADDITIVES ON SLIT PEA CURVATURES IN 2,4-D*

ADDITIVE	M CONC	CURVATURE IN ADDITIVE	CURVATURE IN WATER	M CONC 2,4-D	CURVATURE IN 2,4-D	CURVATURE IN 2,4-D + ADDITIVE	SYNERGISM**
IAN	3.2×10^{-4}	−134±11	−141±9	1×10^{-7}	−115±9	−133±8	−18
	3.2×10^{-4}	...***	−126±7	3.2×10^{-7}	−20±12	−94±5	−74
	3.2×10^{-4}	...***	−126±7	1×10^{-6}	55±13	−57±9	−112
NAN	3.2×10^{-5}	−114±6	−141±9	1×10^{-7}	−115±9	−158±14	−43
	3.2×10^{-5}	...***	−126±7	3.2×10^{-7}	−20±12	−90±7	−70
	3.2×10^{-5}	...***	−126±7	1×10^{-6}	55±13	67±7	12
CHAA	3.2×10^{-4}	−137±12	−162±10	1×10^{-7}	−141±9	−123±10	18
	3.2×10^{-4}	−137±12	−162±10	1×10^{-6}	36±21	−95±13	−131
PBA	1×10^{-5}	−150±12	−162±10	1×10^{-7}	−141±9	−132±11	9
	1×10^{-5}	...***	−126±7	3.2×10^{-7}	−20±12	−16±14	4
	1×10^{-5}	−150±12	−162±10	1×10^{-6}	36±21	28±28	−8

*, **, and *** As in table I.

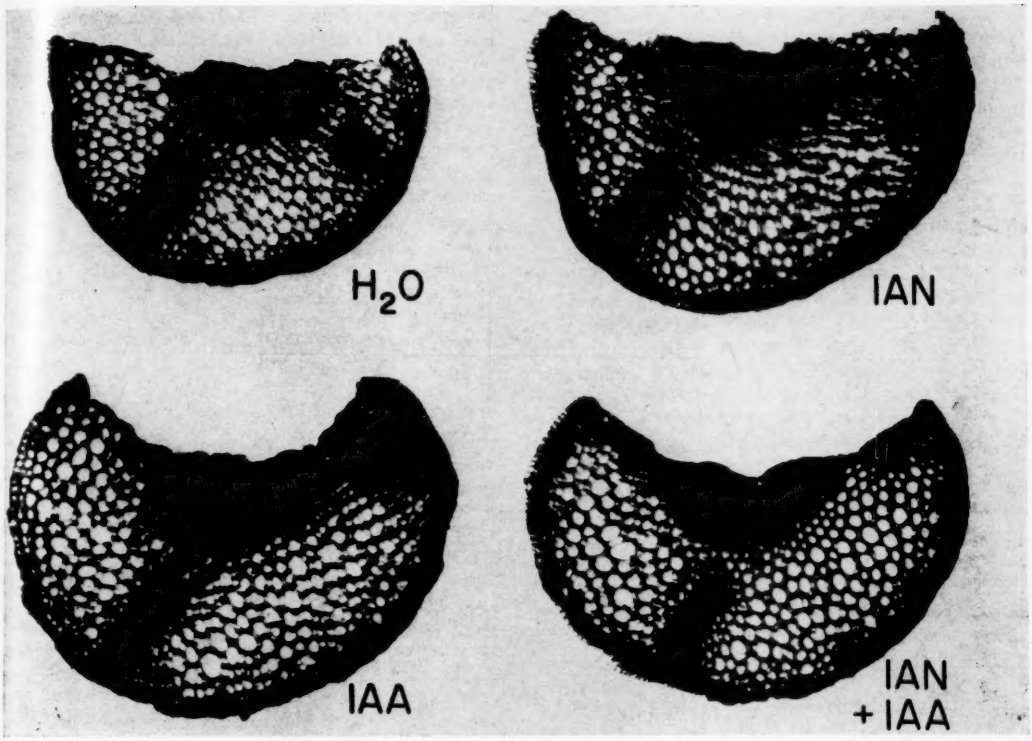

FIG. 1. Freehand cross sections from regions of maximum curvature of slit third internodes of etiolated peas following 24 hour treatment with water, 3.2×10^{-4} M IAN, 1×10^{-6} M IAA, or a combination of the latter two.

in synergism of curvature, alone and at high enough concentration to produce an effect, it reacted differently. Rather than reducing negative curvature as did IAN, 3.2×10^{-4} M NAN markedly increased negative curvature (table I). This difference in response to high concentrations of IAN and NAN does not seem to bear on the main problems of this paper and will not be considered further.

At a concentration of 10^{-4} M, though not at 10^{-5} M, PBA possessed activity in the slit pea test. This was verified in two additional experiments not shown in table I. Attention is called to these results because of reports and discussion by Went (19, 20) and Thimann and Schneider (16) concerning the activity of PBA in the slit pea test.

In connection with the second objective, a brief survey employing freehand sections (fig 1) showed that sections developed a more pronounced crescent shape in the presence than in the absence of IAA. Within the variation that was observable, IAN had no effect that could be distinguished. Perhaps an amount of work seemingly incommensurate with the value of possible results could show an effect of IAN. This was not attempted.

Consideration of the above did suggest that the increases in volume and cross-sectional area of slit sections should be compared with the increases in curvature and length, and that not only slit sections should be measured but also whole sections. The results for IAN, IAA and their interaction are reported in table IV.

Although 20 mm sections slit 17 mm were used and curvatures are reported as curvature changes, the pattern of IAN-IAA synergism in slit pea stem curvature previously reported (9) is still in evidence. Changes in length and weight of slit sections reflect a complete absence of IAN-IAA synergism in these types of growth. In fact, the length and weight changes of slit sections are exceptionally uniform, both in the presence and absence of IAN, over the concentration range of IAA from 10^{-6} to 10^{-5} M.

IAN did show slight effects when administered alone, particularly with whole sections. For whole sections these effects of IAN persist in the presence of 10^{-6} M IAA; but, for slit sections and for whole sections at higher IAA concentrations, no influence of IAN is discernible.

Although slit sections did not elongate as much as whole sections, they did gain as much in weight. This points to a greater increase in cross-sectional area in slit than in whole sections. The magnitude of this difference is shown by the calculated areas.

The increases in cross-sectional area of whole sections were small, and the largest would have necessitated only a 4% increase in average diameter. Actually, microscopic measurement of the shadowgraphs of the sections showed that diameter increase was not uniform and that the apical ends of the sections in IAA and in IAN plus IAA increased in diameter approximately 12%. The apical ends in water and in IAN and the distal ends in all solutions showed insignificant diameter changes.

Cross-sectional area calculations also showed little change in lateral dimensions of slit sections in water and in IAN. In the presence of IAA plus or minus IAN these sections enlarged appreciably in lateral dimensions, but with no hint of synergism. These results are consistent with the changes in cross-sectional shapes of slit sections pictured in figure 1.

Because of the lack of IAN-IAA synergism in length and weight changes under the experimental conditions used in collecting the data of table IV, two changes that could be expected to maximize any differences that might exist were introduced. First, 10 rather than 20 mm sections were used. Such sections are within the region of maximum curvature in the slit pea test, show greater elongation responses than segments farther from the apex, and are large enough to be weighed individually with reasonable accuracy. Second, NAN rather than IAN was used as the synergist. The greatest synergism in curvature found occurred with NAN (table I).

The results appear in table V. Although not directly comparable with those of table IV because of difference in section length and additive, they do confirm the lack of synergism in length and weight changes reported there. In no instance, except curvature, was there any significant effect of NAN.

Discussion

The results presented in tables I and II strongly reinforce the doubt expressed earlier (9), and based on other evidence presented then, that IAA oxidase can be involved to a significant extent in IAN-IAA synergism in the slit pea test.

Bennet-Clark and Wheeler (1) have reported two interesting properties of IAN, namely the inhibition of IAA uptake and of IAA conversion to its aspartate by potato slices. The author reported (9) the apparent reduction in IAA loss from solutions in contact with slit pea internodes in the presence of IAN. This did not occur with whole sections. Reduction by IAN of IAA uptake in slit pea stems is possible, however, if appreciable, it would be most difficult to understand the lack of effect of IAN on elongation and weight increase in such sections. The synergistic properties of IAN could hardly be accounted for by its inhibition of IAA conversion to the aspartate. How could one then account for the synergism of IAN with NAA? The lack of fundamental knowledge concerning the mechanism of auxin action in growth and curvature responses certainly hampers attempts to explain the action of synergists of auxin.

The fact that 2,4-D did not synergize with any of the additives used in no way detracts from the arguments based on similarity of responses found with NAA and IAA. The results might be interpreted as indicating a fundamental difference in the mode of action of 2,4-D. Other interpretations are possible also; but in view of the questions to be raised con-

Table IV

Effects of IAA & IAN on Curvature, Length, Weight, & Cross-Sectional Area of 20 mm Pea Stem Sections*

Measurement	Type of sections	3.2×10^{-4} M Conc IAN	M Conc IAA			
			0	10^{-6}	2×10^{-6}	10^{-5}
Curvature change	Slit	−	−42	56	108	156
		+	−23	110	148	158
% Increase in length	Whole	−	6	20	26	30
		+	10	25	24	28
	Slit	−	2	16	15	17
		+	4	15	17	15
% Increase in weight	Whole	−	8	25	32	38
		+	13	35	34	37
	Slit	−	6	35	38	38
		+	9	33	35	32
% Increase in cross-sectional area	Whole	−	2	4	4	5
		+	3	8	7	7
	Slit	−	4	16	20	18
		+	4	16	15	15

* Sections pooled; therefore S.E. not calculated. $n = 10$ except for slit section curvature and length, where $n = 20$. Average initial values as follows: length (whole & slit) = 20.8 mm; weight (whole) = 57.5 mg; weight (slit) = 55.9 mg; x-sect. area (whole) = 2.8 mm²; and x-sect. area (slit) = 2.7 mm².

cerning the meaning and value of slit pea test results, it seems unwise to use such data (tables I–III) for this purpose.

The most interesting findings of the present work are the complete lack of synergistic interactions in growth of slit sections by elongation or weight gain and the absence of correlation between these and degree of curvature. The latter phenomenon will be considered first.

The pea cylinder shows a marked response to 10^{-5} M IAA. Although the curvature of slit sections changes little at this concentration, appreciable elongation of the arms does occur (9). From 10^{-6} to 10^{-5} M IAA neither whole nor slit sections elongate appreciably, but curvature of slit arms increases greatly. Curvature results from differential elongation of the inner and outer edges of the slit sections (see fig 1). Because of the thinness, small differences in elongation produce appreciable curvature differences [see fig 10 (9)]. It is, then, the balance of elongation between the cells of the inner and outer arcs of curvature rather than total elongation that is important in the slit pea test. Lateral enlargement probably plays an important role also (tables IV & V). Since the growth of any cell in a mass is influenced by the pressures and tensions created by surrounding cells, any lateral enlargement that occurs must be a factor in determining the final curvature attained.

The mechanism of the slit pea test has been discussed at some length (15, 17, 19). Differential elongation and possible reasons for its occurrence have been emphasized, but no satisfactorily complete explanation has been given. The etiolated third internode of the pea is not anatomically simple [see fig 1 of this paper & fig 178 of Hayward (4)]; nevertheless the growth of whole sections appears to be largely restricted and controlled by the intact epidermis (9), which also seems to place severe limits on the radial enlargement of the sections used (tables IV & V). Slitting eliminates much of the restrictive control of the epidermis, permits considerable radial enlargement, and wounds many cells. All these add to the complexity of the situation. Illustrative of this is the reduced elongation of slit as compared to whole sections, whereas total growth as measured by weight increase is not appreciably different. Until it is possible to describe the responses of slit sections to auxins with greater accuracy, and to explain the presence or absence of correlations among elongation, weight, and curvature changes, it is certainly premature to attempt to understand the effects of the synergists; moreover the meaning and value of any results obtained via the curvature test must remain questionable.

Lest the lack of response to IAN and comparable additives in elongation and weight gain be interpreted as absence of effect, it must be emphasized that, at appropriate concentrations of IAA or NAA, cells of the outer arc of curvature do elongate more in the presence than in the absence of the additives. How or why is not clear, though, as mentioned above, lateral enlargement of cells is likely to be important. The lateral enlargement of cells of slit sections is stimulated by IAA. IAN need but modify the polarity of enlargement slightly to exert the observed effects. In whole sections the epidermis may prevent sufficient radial expansion for the expression of this phenomenon.

TABLE V

EFFECTS OF IAA & NAN ON CURVATURE, LENGTH, WEIGHT, & CROSS-SECTIONAL AREA OF 10 mm PEA STEM SECTIONS*

MEASUREMENT	TYPE OF SECTIONS	3.2×10^{-5} M CONC NAN	M CONC IAA 0	M CONC IAA 10^{-6}
Curvature change	Slit	−	8±10	113±8
		+	−12±13	177±8
% Increase in length	Whole	−	4	38
		+	3	42
	Slit	−	3	25
		+	4	26
% Increase in weight	Whole	−	10	48
		+	10	52
	Slit	−	5	42
		+	6	40
% Increase in cross-sectional area	Whole	−	5	6
		+	6	6
	Slit	−	2	13
		+	2	11

* All S.E. <2 except for curvature change, where as indicated. $n = 10$ except for slit section curvature and length, where $n = 20$. Average initial values as follows: length (whole & slit) = 10.7 mm; weight (whole) = 21.0 mg; weight (slit) = 20.4 mg; x-sect. area (whole) = 1.96 mm²; and x-sect. area (slit) = 1.91 mm².

In this latter connection it seems appropriate to refer to the work of others. Osborne (11) used sections cut from the same region of the third internode as those used in this study and reported that, for as long as 24 hours in solutions of IAA up to 5 ppm (2.9×10^{-5} M), increase in length was directly proportional to increase in fresh weight. This indicates no increase in diameter in whole sections and corresponds closely to what has been reported here.

Sections cut closer to the apex do not retain their original lateral dimensions, but increase greatly in cross-sectional area in response to IAA. For example, calculations using the data of Galston, Baker, and King (2) show that cross-sectional area increased 53 % more in 5.7×10^{-5} M IAA than in water. Such sections also differ in other ways from those taken farther from the apex (12).

Summary

IAN, NAN, CHAA, and possibly PBA synergize with IAA and NAA, but not with 2,4-D in the slit pea curvature test. Skatole and indole do not. Certain concentrations of IAN, PBA, and NAN, alone, are active in this test. The first two decrease negative curvature; the last increases it.

Because of the synergistic interactions involving NAA, it seems possible to exclude IAA oxidase from further consideration in the phenomenon of synergism in the curvature test.

Slitting the pea internode markedly reduces its ability to elongate but does not appreciably lower its capacity to increase in weight and, therefore, volume. These measures of growth do not show synergistic interactions between IAA and IAN or NAN. In these respects, slit sections respond much as do whole sections.

Curvature does not reflect average elongation nor weight gain, but the differential in elongation between cells of the outer and inner regions of the arc of curvature. The degree of curvature must also be influenced by the lateral enlargement of cells. In the internode region used, lateral enlargement is appreciable in slit sections but not in whole.

IAN, NAN, CHAA, and PBA may somehow alter the growth responses of cells, possibly by modifying polarity. In so doing the curvature responses of slit pea internodes are changed. These compounds, however, cannot be considered to interact synergistically with auxins in growth as measured by total cell elongation or enlargement.

The interpretation of the slit test is more complex than that of the cylinder test. The slit internode does curve in response to auxins, but curvature is no guarantee of auxin activity. Until correlated with other tests, the meaning of activity in the slit pea test must be questioned.

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EFFECTS OF CALCIUM, POTASSIUM, & MAGNESIUM ON OXALIC, MALIC, & CITRIC ACID CONTENT OF VALENCIA ORANGE LEAF TISSUE[1]

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The importance of organic acids in plant metabolism is well established. Several investigations have dealt with the effect of nutrition on organic acids of leaf and fruit tissues (2, 3, 5, 13). In general, the effects of cations on the organic acids in leaf tissue are rather complex. For example, Cooil (1) found that the higher total acids associated with high K in expanding guayule leaves was largely accounted for by high citric acid levels, whereas in mature leaves high malic acid levels were associated with high Ca levels. In tung, oxalic acid formation is directly related to nitrate and Ca supply (3).

Citric acid is the main acid in citrus fruits (8, 9) and the amount in the fruit is influenced by K nutrition (11). Approximately twice as much malic as citric acid, however, is found in citrus foliage and oxalic acid as insoluble Ca-oxalate occurs in even greater concentrations than malic acid in citrus foliage (10).

The present study was concerned with a quantitative determination of oxalic, malic, and citric acids in Valencia orange leaf tissue grown with a wide range of Ca, K, and Mg. The objective was to determine what effect any one of the cations may have on the accumulation of the three acids in the leaf.

Methods & Materials

For this study 4- to 5-month-old Valencia orange foliage collected from trees in a sand culture experiment (11) showing a wide range of Ca, K, and Mg levels was used for organic acid analyses. Leaf-sample preparation and methods of Ca, K, and Mg determination are described elsewhere (7).

The methods used for H_2O-extracts of the leaf tissue and for separating malic and citric acids on ion exchange columns were similar to the methods described by Palmer (6) for analyses of tobacco leaves. A 500 mg sample of the powdered tissue was extracted with H_2O on a steam bath, filtered, and made up to 25 ml in a volumetric flask. Three milliliters of the extract were placed on top of Dowex 1-X4 50 to 100 mesh ion exchange resin in a chromatographic tube. The resin was previously converted to the formate form by washing with 1 N sodium formate. The column was then washed with two volumes of H_2O to remove the neutral substances. After the flow rate was established with H_2O, formic acid in increasing strengths was passed through the column to elute and separate the H_2O soluble organic acids. The formic acid was added to the column by an automatic mixing device described by Palmer (6). Consecutive 3 ml fractions were collected from the columns and evaporated to dryness at temperatures below 60° C on a hotplate to remove the formic acid. After being dried, the acids were dissolved in 2 ml of H_2O and titrated with 0.01 N NaOH to the phenol red endpoint. The phenol red indicator was prepared by dissolving 0.1 g of dry indicator in 28 ml of 0.01 N NaOH and diluting to 250 ml.

The identities of malic and citric acids were determined by adding known amounts of the acids to the ion exchange columns with and without the leaf extracts followed by titration to ascertain which fractions contain malic and citric acids. Malic acid came off the columns in fractions 5 to 11 and citric in fractions 14 to 19.

Total H_2O-soluble acids were eluted from the ion exchange columns with 6 N formic acid and then determined by titration by the same methods as for malic and citric acids.

The method of Baker modified by Palmer was used with minor changes for oxalic acid determination (6). Ten milliliters of 1 N HCl were added to 250 mg of dry leaf tissue, heated for 15 minutes in a steam bath, allowed to cool, and made up to 25 ml volume. After standing overnight, 5 ml of PO_4-tungstate reagent were added to a 5 ml aliquot of leaf extract and allowed to stand 5 hours to precipitate the nitrogenous organic compounds. Then the extract mixture was centrifuged at 1500 g for 10 minutes. Next, a 5 ml aliquot was added to another centrifuge tube and made alkaline by adding 6 N NH_4OH drop by drop until a slight precipitate of PO_4-tungstate was formed. Three milliliters of a $CaCl_2$-acetate buffer were added to each tube and, after standing overnight, the extract was centrifuged again. The supernatant was decanted and the precipitate was washed with 5 % acetic acid saturated with Ca-oxalate, centrifuged, the supernatant was decanted, and then the Ca-oxalate precipitate was dissolved in 5 ml of hot 4 N H_2SO_4 and titrated with 0.02 N $KMNO_4$.

Results & Discussion

Effect of Ca: The sand culture experiment from which the leaf samples were taken contained trees with five levels of leaf Ca while K was relatively constant at about 1.2 % K in the dry matter of 4- to 5-month-old leaves. In this set of samples Ca reciprocated with Mg; as leaf Ca increased from about 1.5 to about 3.2 %, leaf Mg decreased from 0.87 to about 0.25 %.

Figure 1 illustrates the effect of Ca on oxalic, malic, citric, and total H_2O-soluble acids. Each point on the graph represents the average of four replications for each of the five Ca levels. The range of oxalic acid was from 0.40 to 0.98 meq/gm dry weight while Ca increased from 1.47 to 3.16 %. The coefficient of correlation (r) between Ca and oxalic

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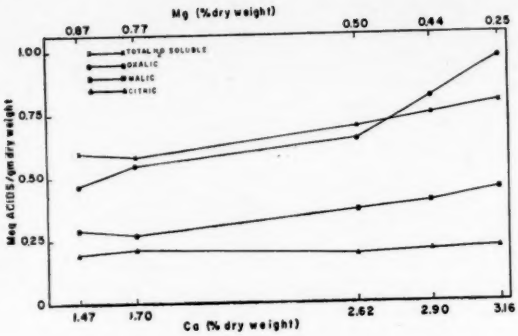

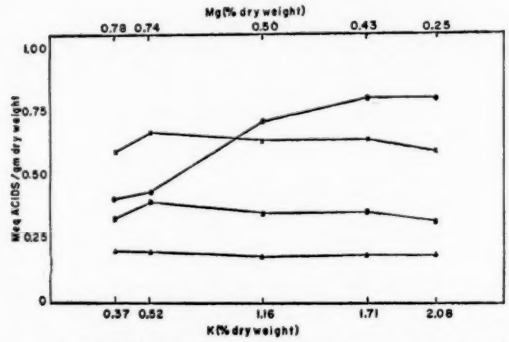

FIG. 1. Effect of Ca on oxalic, malic, citric, and total H_2O-soluble organic acids when Mg is the reciprocating cation.

FIG. 2. Effect of K on oxalic, malic, citric, and total H_2O-soluble organic acids when Mg is the reciprocating cation.

acid is highly significant as shown in table I. Since oxalic is found as Ca-oxalate, which is insoluble in citrus foliage, the increase of oxalate in the high-Ca leaves probably has no effect on the total organic acid metabolism. However, the formation of Ca-oxalate could be at the expense of other metabolites. This does not seem to be the case; at least malic acid, which is the major active organic acid in citrus foliage, also increased with increasing Ca. Malic acid levels ranged from 0.28 to 0.43 meq/gm from the low to high level of Ca. The r value was 0.7, which is highly significant (table I). The malic acid levels were lower on the average than those found by other workers (9), probably because of younger leaves and different methods of leaf-sample preparation. The leaves in the present study were 4 to 5 months old as compared with 1 year in the other study. Young leaves generally have a higher respiration rate than old ones; therefore from time of collecting to drying more malic acid could have been lost in our samples. Vickery et al (14) found a very rapid decrease in malic acid in the dark with a corresponding increase of citric in tobacco tissue. Nevertheless an effect of Ca on malic acid level is still evident in the present study.

Citric acid levels ranged from 0.18 to 0.23 meq/gm of dry weight in these leaf samples. However, no trend was apparent in relation to Ca levels and the r value was not significant.

The total H_2O-soluble acids also increased as the Ca level increased primarily because of the increased level of malic acid. Malic and citric made up 75 to 90 % of the total H_2O-soluble acids. The other 10 to 25 % could be accounted for by other organic acid radicals plus some inorganic acid radicals; however, usually it takes more severe conditions to remove inorganic acid radicals from chromatographic columns than those used here.

EFFECT OF K: In another part of the same sand culture, Valencia orange trees contained five levels of K while Ca was held relatively constant between 2.5 and 3.0 %. In these samples K reciprocated with Mg.

Figure 2 illustrates the effect of K on total H_2O-soluble, oxalic, malic, and citric acids when Mg was the opposed variable. Each point represents the average of four replications. As K increased from 0.37 to 2.08 %, oxalic acid was the only acid to increase in concentration, and it increased from 0.41 to 0.81 meq/gm dry weight. Any change in malic, citric, or total H_2O soluble acids was small and inconsistent; therefore the r values in table I are not significant. The r value showing the relation between K and oxalic is highly significant.

Oxalic acid was found as the insoluble Ca-oxalate even though it was increased in the foliage by increased K. The function of Ca that K was performing is not known, but Ca must have been released from some other reaction or function in order for increasing amounts to be used for the neutralization of oxalic acid. At the lowest K level, about 15 % of the leaf Ca was tied up as oxalate. At the higher K levels, about 30 % of the Ca was in the oxalate form.

Citric acid is the major acid of citrus fruit (8, 9) and its level is affected by the K level in the tree.

TABLE I

CORRELATION COEFFICIENTS SHOWING ASSOCIATIONS BETWEEN Ca, K, & Mg & OXALIC, MALIC, CITRIC, & TOTAL H_2O-SOLUBLE ORGANIC ACIDS OF VALENCIA ORANGE LEAF TISSUE

VARIABLE CATION	CONSTANT CATION	RECIPROCATING CATION	OXALIC ACID	MALIC ACID	CITRIC ACID	TOTAL H_2O-SOLUBLE ACIDS
Ca	K	Mg	0.81	0.71	0.11	0.76
K	Ca	Mg	0.88	−0.19	−0.26	−0.22
Mg	K	Ca	−0.82	−0.69	−0.25	−0.64
Mg	Ca	K	−0.87	0.23	0.36	0.24

r* (0.05) = 0.44, 0.01 = 0.56

r* — Correlation coefficient.

However, K level had no effect on citric acid in the leaf tissue as shown in table I and figure 2. Other workers (4) reported finding enzyme systems in fruit vesicles capable of producing citric acid and still others (10) have found very little citric acid in the peel. These results make it extremely doubtful that the citric acid produced in the foliage is transported to the fruit. It is not known why K influences the citric acid metabolism of the fruit and not that of the foliage.

EFFECT OF Mg: The data presented for Ca and K involved Mg as an opposed variable. The correlations and trends in table I therefore are, as would be expected, almost the reverse of those shown for Ca and K. By considering these data alone, one might conclude that all the effects found were induced by Mg. However, data from analyses of other citrus leaf samples show that Ca is the dominant cation influencing organic acid production, particularly oxalic.

Under normal conditions Ca reciprocates mainly with K in the leaf tissue, although Mg is somewhat of a factor when K is low (12). Analyses of citrus leaf tissue in which Ca increased from 1.0 to 3.6 % show that oxalic acid increased from 0.20 to 0.80 meq/g dry weight. Mg was relatively constant between 0.31 and 0.38 % of the dry weight and therefore would have no effect on the oxalic acid content. Malic acid also increased with increased Ca in these samples.

When Ca is very high in the soil or solution cultures, the Ca level in the leaf tissue may increase without base reciprocation (12). A total base constancy of about 200 meq/g dry leaf tissue exists until Ca reaches 3.5 %. Above 3.5 % Ca the total base content increases as Ca increases. Oxalate content as high as 1.2 meq/g dry weight was found in leaf tissue with Ca levels between 4.0 and 4.5 % and relatively constant Mg levels of 0.37 to 0.39 %. Malic acid levels were also high in these samples. Therefore, the negative correlations between Mg and oxalic and malic acids in table I seem to be a result of experimental design and not of an effect of Mg.

SUMMARY

Valencia orange leaves collected from a sand culture experiment which showed a wide range of Ca, K, and Mg were analyzed for oxalic, malic, citric, and total H_2O-soluble organic acids. Oxalic acid was determined chemically with final titration in hot H_2SO_4 with 0.02 N $KMNO_4$. Malic, citric, and total H_2O-soluble organic acids were separated from H_2O extracts on ion exchange columns containing Dowex 1-X4 resin in the formate form and titrated to the phenol red endpoint.

Oxalic acid increased from 0.41 to 0.98 meq/g while Ca increased from 1.5 to 3.2 % of the dry weight with K relatively constant. When K increased from 0.37 to 2.08 % with Ca relatively constant, oxalic acid increased from 0.40 to 0.81 meq/g.

Malic acid was positively correlated with Ca; however, the increase in malic was not as great as the increase in oxalic acid. Malic acid concentration was not influenced by K.

Citric acid was virtually constant in all samples ranging only from 0.18 to 0.23 meq/g of dry matter. No trend or correlation existed between citric acid and the three cations.

The total H_2O-soluble organic acids were affected only by Ca, primarily because of increased malic acid in presence of increased Ca levels of the foliage.

Mg as a reciprocating cation showed opposite effects from those of both K and Ca, but these appear to be coincidental with no primary relation between leaf Mg and the three organic acids.

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OLIGOSACCHARIDE PRODUCTION FROM SUCROSE BY VERTICILLIUM ALBO-ATRUM[1,2]

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During the course of a study on the component sugars of the mycelium of *Verticillium albo-atrum*, Reinke and Berth. (9), several unidentified sugars were detected in the culture filtrates when sucrose served as the carbon source. Although many workers have studied the utilization of various carbohydrates by this fungus, little is known about its carbohydrate metabolism. It was therefore of interest to characterize more fully these unknown sugars. Some of these results have previously been reported in abstract form (8).

MATERIALS & METHODS

The composition and preparation of the basal medium and the procedure for inoculation have been described previously (9). Eighteen to twenty-four flasks of medium were prepared for each experiment and the contents of three flasks were collected at 3 to 4 day intervals after inoculation. The amount of mycelium (mg) produced was determined by collecting the mat from each flask in a sintered glass crucible. The mat was then rinsed with distilled water and dried overnight at 90 to 95° C. The filtrates and washings were combined and the pH was determined (glass electrode) and adjusted to 6.5 to 7.0 if necessary. Three volumes of 95 % ethanol were added to the filtrate and the mixture was boiled 10 minutes under reflux. Any precipitate that formed on cooling was removed by filtration. The alcoholic mixture was concentrated under reduced pressure to a volume of 5 to 15 ml, depending on how long the fungus had grown in the medium. Sufficient ethanol to yield a final concentration of 20 % ethanol was added as a preservative.

The concentrated filtrates were chromatographed (descending one dimensional) on Whatman no. 1 paper with the organic phase of one of the following mixtures as the irrigating solvent: phenol-water (adjusted to pH 5.0–5.5), ethyl acetate-pyridine-water (8/2/1, v/v/v) or butanol-acetic acid-water (4/1/5, v/v/v). The latter irrigant was particularly effective for separating the sugars present in the medium. The sugars were located on the dried chromatograms with various spray reagents, including 0.1 % resorcinol in 0.5 N HCl, aniline hydrogen phthalate and 3,5-dinitrosalicylic acid (3). Available known sugars were included on the same chromatograms to aid in the identification of sugars in the filtrates.

RESULTS & DISCUSSION

A typical growth curve for *V. albo-atrum* grown on the liquid medium and the changes of the pH of the medium have been published previously (9). In general, with 3 % sucrose as the carbon source there is little growth for the first few days. This is followed by a rapid increase in mycelial mat weight until a maximum is reached about 15 days after inoculation. The culture then begins to lyse and the medium becomes increasingly alkaline. The exact nature of the growth curve in this medium will vary somewhat depending on the temperature, the initial pH of the medium, and the isolate of fungus used.

After 3 to 4 days growth, sucrose was the major sugar in the medium but fructose, glucose, and an unknown, A, also were present. Unknown A was detected with the resorcinol reagent and had an $R_{sucrose}$ of 0.55 in butanol-acetic acid-water. After 7 days the sucrose concentration decreased considerably. Glucose, fructose, unknown A, and two new unknowns, B and C, were also detected with the resorcinol reagent. The $R_{sucrose}$ values for unknowns B and C in butanol-acetic acid-water were 0.31 and 0.17, respectively. By about 11 days after inoculation small quantities of sucrose, fructose, glucose, and unknown A were usually detected but spots due to unknowns B and C were absent. After approximately 15 days growth the sucrose was exhausted and sugars were no longer detected in the medium.

In addition to the presence of ketoses as indicated by the reaction with resorcinol, unknowns A, B, and C were non-reducing and did not react with the molybdic acid reagent for phosphate (3). Only fructose and glucose were detected when the concentrated medium or the unknowns eluted from the chromatogram were hydrolyzed and rechromatographed. A visual comparison of spot size and intensity on the chromatograms of hydrolyzed eluates showed that there was more fructose than glucose in the unknowns. From this information and from the mobility of the unknowns on chromatograms, it appeared that unknowns A, B, and C were oligosaccharides similar to those produced by enzyme preparations from other fungi (2, 10). A straight line was obtained when the logarithms of the distance traveled

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by the unknowns in butanol-acetic acid-water were plotted against assumed glucose-fructose ratios (fig 1). Such a linear relationship has been found to exist between the mobility and molecular size of a homologous series of oligosaccharides (12). This evidence indicated that unknowns A, B, and C were a trisaccharide, tetrasaccharide, and pentasaccharide, respectively.

Attempts were made to obtain more of these compounds so that the glucose-fructose ratio could be determined. Experiments showed that the production of the oligosaccharides was dependent on the sucrose concentration in the medium. Adding more sucrose also increased the mycelial growth and the length of time before lysis began. When initial concentrations of 15 or 20 % sucrose were used, large quantities of unknowns A and B and lesser quantities of unknown C and another material, apparently a hexasaccharide, were found in the medium. Estimates based on spot size and intensity of the sprayed chromatogram indicated that 1 to 2 % of unknown A may be present at times in this medium.

The concentrated medium containing large amounts of the unknowns A and B was applied to large sheets of Whatman no. 1 filter paper and irrigated for 5 days with the butanol-acetic acid-water mixture. Side strips were then sprayed with the resorcinol spray to locate the unknowns. The unknowns were eluted with water and the eluate hydrolyzed with 0.05 N H_2SO_4 for 1½ hours. The H_2SO_4 was removed by adding solid $BaCO_3$ until the liquid was slightly acid (pH 4–5), and the hydrolyzate concentrated under reduced pressure. The hydrolyzate was chromatographed and the amounts of glucose and fructose were determined by the method of Koch et al (7). The fructose to glucose ratio of unknown A was found to be 1.9 to 1 while the ratio for unknown B was 2.8 to 1. These values are 95 and 93 % of the theoretical values of 2 to 1 and 3 to 1 for a trisaccharide and tetrasaccharide. Further, co-chromatography in butanol-acetic acid-water showed that unknowns A and B were indistinguishable from a sample of tri- and tetrasaccharide produced by an enzyme preparation from *Aspergillus oryzae* (10).

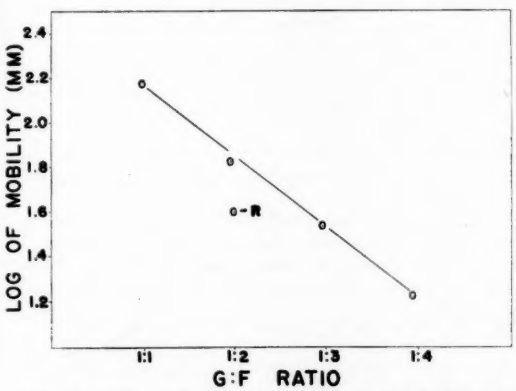

FIG. 1. The plot of the logarithm of the mobility of unknown sugars versus assumed glucose-fructose ratios. R indicates the mobility of the trisaccharide, raffinose.

Preliminary experiments have shown that a trisaccharide and tetrasaccharide are formed when dialyzed cell-free extracts of *V. albo-atrum* are incubated with sucrose. The production of the oligosaccharides took place in the absence of added phosphate. It thus appears that the production of these oligosaccharides is due to transglycosylation of fructose (2, 5, 10).

These oligosaccharides have not been found in the medium when 3 % fructose, 3 % glucose, or a mixture of 1.5 % glucose and fructose was used as the carbon source. Two reducing compounds believed to be a tri- and tetrasaccharide have been detected in the medium when maltose was used as a carbon source.

In addition to the oligosaccharides, small quantities of three other unknowns were sometimes observed on the chromatograms. These unknowns appeared in the medium during the period 7 to 15 days after inoculation. Unknown D has an $R_{fructose}$ of 1.6 to 1.7 in butanol-acetic acid-water and is lost when the chromatograms are irrigated for more than 48 hours with this solvent. It is a non-reducing material that reacts with the resorcinol reagent. Unknown E is a non-reducing material that reacts with resorcinol and was originally thought to be raffinose (8). Further work has made this identification questionable. The color with the resorcinol spray is not the same as with known raffinose. In addition, if chromatograms are irrigated for 96 to 120 hours with butanol-acetic acid-water, it is less mobile than known raffinose. Other experiments have shown raffinose can be utilized as a carbon source by the fungus. Thus this unknown would probably be metabolized from the medium if it were raffinose. Unknown F is a reducing compound that reacts with the resorcinol spray and has an $R_{sucrose}$ of about 0.75 in butanol-acetic acid-water. This material is found in greater concentration if the initial sucrose concentration of the medium is 10 to 15 %. These unknowns may arise from enzymatic transfer of fructose to some non-sugar moiety (1) or they may be products of sugar catabolism.

All 16 isolates of Verticillium tested have been found to produce the trisaccharide and tetrasaccharide from sucrose. These isolates included pseudo-sclerotial, dark mycelial and white mycelial types and were obtained from various parts of the United States. It has been stated that the dark mycelial types should be called *Verticillium albo-atrum* and the pseudosclerotial types should be named *Verticillium dahliae* Kleb. (11). However, the term *V. albo-atrum* is used for both types in this study since the workers who furnished the isolates used this designation. Thirteen of the isolates were originally isolated from potato plants and the remaining three were obtained from pepper, maple, and tomato. While all these isolates were not compared in one experiment, it appears there are differences in the ability to produce oligosaccharides. Some isolates hydrolyze the su-

crose to glucose and fructose more rapidly and the amount of oligosaccharides produced is low.

The production of the oligosaccharides was demonstrated in other liquid media in which sucrose is the carbon source. Of several media investigated, one was particularly good. In addition to sucrose, the medium is composed of—asparagine monohydrate—1.2 g, KH_2PO_4—1.0 g, $MgSO_4 \cdot 7H_2O$—0.5 g, and the same concentration of iron and minor elements used with the original medium. The amount of growth and the production of oligosaccharides are dependent on the concentration of sucrose. This medium does not become alkaline (initial pH 4.5) and there is no lysis for at least 35 days if 5 % sucrose is used as the carbon source. In addition the oligosaccharides appear in greater concentration and are present over an extended period of time. Analysis of the culture filtrate showed the presence of the tri- and tetrasaccharides from about eight days after inoculation and they were still found after 30 days of growth. Appreciable quantities of the pentasaccharide and smaller amounts of hexasaccharide were detected after about 20 days growth and were still present after 30 days of growth. It therefore appears that attempts to isolate and characterize these unknowns more fully might most conveniently be made with this medium.

Various investigators have reported the presence of polysaccharide material in culture filtrates of *V. albo-atrum*. In some instances these materials are found in quantity only after lysis has begun (6, 9). In other instances, however, the polysaccharide materials appear during the early phases of growth (4). Results in the present study indicate that the production of polysaccharide material in the early phases of growth may be dependent upon the composition of the medium and the isolate of fungus used.

Analyses of the alcohol insoluble portion of the culture filtrate and of the mycelium have not shown any detectable oligosaccharide beyond the hexasaccharide. As the higher oligosaccharides occur in the filtrate only with a high sucrose concentration, it does not appear that polysaccharide material is built up solely by the transglycosylation reaction. It does appear that fructose can be transferred to other materials in the alcohol insoluble fraction and the mycelium. Thus, fructose has been detected chromatographically in hydrolyzates of the alcohol insoluble portion of the filtrate and of the mycelium collected during the early phase of growth. Since fructose was not detected in the mycelium after lysis began (9), the fructose present in these fractions during the early phases of growth is probably reutilized.

Summary

An homologous series of non-reducing oligosaccharides was found in the culture filtrates when *V. albo-atrum* was grown on media containing sucrose. Increasing the initial sucrose concentration increased the number and concentration of the oligosaccharides and the length of time they occur in the filtrates. The oligosaccharides appear to be formed by enzymatic transfer (transglycosylation) of fructose.

Acknowledgment

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SOME EFFECTS OF TONICITY ON LUPINE SUCCINOXIDASE[1]

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The integrity of mitochondria is relevant to the intracellular distribution of inorganic ions by Donnan equilibria and active transport processes (cf. 1–4, 17, 25, 31, 37, 39, 41, 42, 47). Two related properties of mitochondria probably dependent upon their integrity and especially relevant to ion distribution are the permeabilities to salts, sucrose, and organic acids (2, 6, 9, 20, 34) and the ability of mitochondria to act as osmometers (26, 27, 35, 43, 44). Furthermore, since respiratory activity is influenced by the osmolarity of the media used in both preparation and assay procedures (8, 13, 14, 18, 21–23, 40, 45) the osmotic properties of mitochondria also must be considered with the effects of inorganic ions not only upon ion uptake but also with the effects of ions upon the oxidation of cytochrome c (18, 28, 36, 46), reduced diphosphopyridine nucleotide (18) and various organic acids (30).

As Tyler (45) pointed out, few investigators have considered tonicity itself to affect the activity of mitochondrial enzymes, apart from any osmotic influence on permeability or swelling of mitochondria. One inadequacy in noting the effect of different osmotic concentrations on enzymatic activity usually stems from an arbitrary selection of substrate concentration since either inhibition or activation may result depending upon the substrate concentration used (15, 16). A more complete characterization is required, such as the determination of the apparent reaction constants for succinoxidase (cf. 41). A complicating factor in experiments concerning tonicity results from the common use of sucrose as the chief solute for varying osmotic pressure. Lehninger, Ray, and Schneider (24) discuss evidence for the inhibitory action of sucrose on mitochondrial activities. In common with studies on tonicity effects, however, sucrose effects may be incompletely characterized.

In our study concerning the ionic relations of plant mitochondria (cf. 17, 18, 37), information was required to characterize the behavior of lupine mitochondria in different osmotic conditions. Results are reported of experiments studying the effects of both pre-assay and assay tonicities upon the kinetics of lupine succinoxidase under conditions used for studying mitochondrial swelling in the presence of succinate (16).

Methods

Mitochondria were isolated from 4 day-old seedlings of *Lupinus albus* (L.) and washed as previously described (16). The medium used for isolating the lupine mitochondria was 0.165 M in tris(hydroxymethyl)aminomethane (tris), 0.020 M in ethylenediamine tetraacetic acid, and the osmolarity[2] adjusted to 0.15 M, 0.22 M, 0.40 M, 0.60 M, or 0.74 M. Sucrose solutions used to wash the mitochondria corresponded in tonicity to those of the isolation media.

Mitochondrial concentrations were measured by their nitrogen content assayed by the Nessler method.

In the manometric assay for succinoxidase activity the mitochondria suspended in sucrose solution were added to the main compartment from the sidearm of Warburg vessels to start the reaction after a temperature equilibration of 10 minutes. The earliest oxygen uptake data were recorded 3 to 7 minutes after mixing and the uptake followed for 60 minutes. The initial rates of succinoxidase activity were obtained by extrapolating to zero time. Activities were recorded as microliters of oxygen consumed per hour per mg mitochondrial nitrogen [$Qo_2(N)$]. The final assay mixture of 3 ml contained succinic acid, tris, Na_2ATP (Pabst), horse heart cytochrome c (Sigma) as noted, tris-acetate buffer, magnesium acetate as noted, and sucrose to adjust the osmolarity. Although ATP was not required for activity, it increased and prolonged activity. ATP was therefore added routinely in a high saturating concentration. Details of concentrations used are given for each experiment. The succinoxidase assays were carried out around pH 7, which was previously determined to result in maximal activity. Preliminary studies showed the effect of ATP can be attributed to the contamination of ATP by ADP and formation of ADP from ATP. No other 5′-nucleotide was effective.

The terminology of Friedenwald and Maengwyn-Davies (10, 11) is used for classifying the order of reactions. First order reactions concern the complexing of only one molecule each of substrate, activator, or inhibitor with each enzyme site. Second order theory is concerned with cases in which two molecules of any one reactant per site are involved.

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[2] Osmolarities of the media were computed on the basis of the following degrees of dissociation (12): 0.86 for R^+R^-; 0.72 for R^{++} $(R^-)_2$ and $(R^+)_2R^{--}$; and 0.45 for $R^{++}R^{--}$.

The following equations used to characterize lupine succinoxidase have been taken essentially as from Friedenwald and Maengwyn-Davies (10, 11). The Michaelis-Menten equations have been extended to include the effect of an activator and the quantitative relationships between the formation of the various complexes of enzyme, substrate, inhibitor and activator.

The following equations were used as bases for the mathematical representations of the apparent first order reaction of the succinoxidase system in the presence of a dissociable inhibitor malonate:

$$\text{I}\quad E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3'} E+\text{Product}\,,\ K_S = \frac{k_2 + k_3'}{k_1}\ ;$$

$$E+I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI\,,\ K_I = \frac{k_5}{k_4} = \frac{[E][I]}{[EI]}\ ;$$

$$ES+I \rightleftharpoons EIS\,,\ \alpha K_I = \frac{[ES][I]}{[EIS]}\ ;$$

$$EI+S \rightleftharpoons EIS\,,\ \alpha K_S = \frac{[EI][S]}{[EIS]}\ ;$$

where E is succinoxidase, S is the substrate succinate, I is the inhibitor malonate, the combinations of letters are the various complexes, α is a dissociation factor (10) for the dissociation of EIS to ES, EI, S, and I, and the bracketed letters represent concentrations. If α equals one, the inhibition is non-competitive. The complexes of S and I do not affect the complexing of the other on E. The inhibition, thus, affects the enzymatic rate, i.e. influences the enzyme, rather than the affinity of enzyme with substrate (5). If α is infinite, the inhibition is competitive and, in essence, S and I compete for the same site on E. That is, the complex EIS cannot be formed. From steric considerations all values between unity and infinity may be possible for α depending on how close the sites for S and I are together.

In the visual estimation of α from the intersection of the lines of regression, 1/v versus 1/[S] (see later) for [I] and $[I]_2$, values of α around 100 are not easily distinguishable from infinity. An intersection of the lines very near the ordinate may be erroneously assigned to occur on the ordinate. Consequently, values of α and all other constants were calculated from the Lineweaver-Burk double reciprocal plots.

The Michaelis-Menten convention is used in which:
$[E]_t$ equals the sum of [E], [ES], [EI] and [EIS]; v the reaction rate is proportional to [ES]; and V_m the maximal activity is proportional to the total amount of enzyme $[E]_t$. The general equations representing the apparent first order reaction then follow:

$$\text{IIa}\quad \frac{V_m}{v} = 1 + \frac{K_S}{[S]} + \frac{[I]K_S}{[S]K_I} + \frac{[I]}{\alpha K_I}\ ;$$

$$\text{IIb}\quad \frac{1}{v} = \frac{1}{V_m}\left(1 + \frac{[I]}{\alpha K_I}\right) + \frac{K_S}{V_m}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]}\ ;$$

where v is the reaction rate for any given concentrations of S and I, and V_m is the extrapolated reaction rate at infinite S concentration in the absence of I. If 1/v as a function of 1/[S] is determined at two or more different constants [I], all the constants which occur in line 2b (Lineweaver & Burk form of line IIa) can be calculated from the lines of best fit (least squares method). Line IIb yields only apparent values for the constants K_S and K_I if a dissociable activator in the system is not accounted for.

Friedenwald and Maengwyn-Davies (10) have shown that the line IIb has the form following for the first order reaction system involving both a dissociable inhibitor and activator when certain simplifying assumptions are made (see 10):

$$\text{III}\quad \frac{V_m}{v} = \left(1 + \frac{K_S}{[S]} + \frac{[I]K_S}{[S]K_I} + \frac{[I]}{\alpha K_I}\right)\left(1 + \frac{K_A}{[A]}\right).$$

On comparison of line III with line IIa, it is seen that if the effect of a dissociable activator is neglected the values of the various constants from line II are underestimated by $1/(1 + K_A/A)$. Owing to the practical limitation of the number of assays for any mitochondrial preparation, the routine estimations of the apparent first order reaction constants were carried out in the presence of a constant high ATP concentration without any attempt to account for the underestimate of values.

Friedenwald and Maengwyn-Davies (10) pointed out that the values of the various constants are only approximations if the dissociation factors α, x, q, and μ are not equal to unity. x, q, and μ are analogous to α in the dissociations of the various complexes of S, I, A, and E in EAS, EAI, EIS, and EAIS.

For evaluation of the second order reaction constants with respect to substrate K_1/K_2 and v_m the following was used:

$$\frac{v_m}{V} = 1 + k\left(\frac{S_o}{S} + \frac{S}{S_o}\right)$$

where k equals $(K_1/K_2)^{1/2}$, S is the succinate concentration giving the activity V, S_o is the succinate concentration giving the highest experimental activity, and v_m is the extrapolated maximal activity. K_1 and K_2 are the apparent dissociation constants for succinate in the apparent activating and inhibiting positions, respectively, in the relation:

$$\frac{v_m}{V} = 1 + \frac{K_1}{[S]} + \frac{[S]}{K_2}\ .$$

Experimental Results

Initial Observations. Representative data on the effect of the assay osmolarity on succinoxidase activity of lupine mitochondria isolated in hypertonic solutions are shown in figure 1 A. Succinoxidase activity decreased as the osmolarity was progressively lowered below 0.40 M.

Another type of response to osmotic variation is shown in figure 1 B where a peak of maximal activity around 0.40 M was found. In an incomplete survey of experimental conditions, all lupine succinoxidase which characteristically showed the peaked type activity-tonicity curves of figure 1 B also simultaneously displayed second order reactions with respect to succinate concentrations. The peaked type activity-tonicity curve and second order reactions more frequently occurred under hypotonic conditions where the mitochondria may be relatively less intact. It appears that these associated behaviors are related to mitochondrial damage.

Either inhibitions or activations by high osmolarity compared with low osmolarity may result depending upon the substrate concentration used (fig 2). It was apparent that to obtain less equivocal effects of tonicity a more complete characterization was required than that obtained with an arbitrary single concentration of substrate. We therefore determined the apparent reaction constants for succinoxidase from the Lineweaver-Burk double reciprocal plots of data such as in figure 2 (cf. 10).

The data of table I indicate that an oxidation of malate converted from the added succinate was probably negligible and thus complete tricarboxylic acid cycle activity was also negligible with the addition of succinate only. In contrast to Price and Thimann (32) and Pierpoint (29) who obtained R.Q. values of 0.23 to 0.28 with succinate and 1.08 to 1.40 with malate, we obtained generally little carbon dioxide production with succinate even with the system fortified with ATP and DPN (table I). The addition of DPN increased the rate of oxidation of added malate, in accord with the absolute requirement for DPN found by Price and Thimann (32); but the rate initially was nil and very low after 60 minutes. In some experiments an R.Q. up to 0.23 was found with succinate. However, in every case the initial rate of oxidation with a mixture of malate and succinate was equal only to the rate expected from the amount

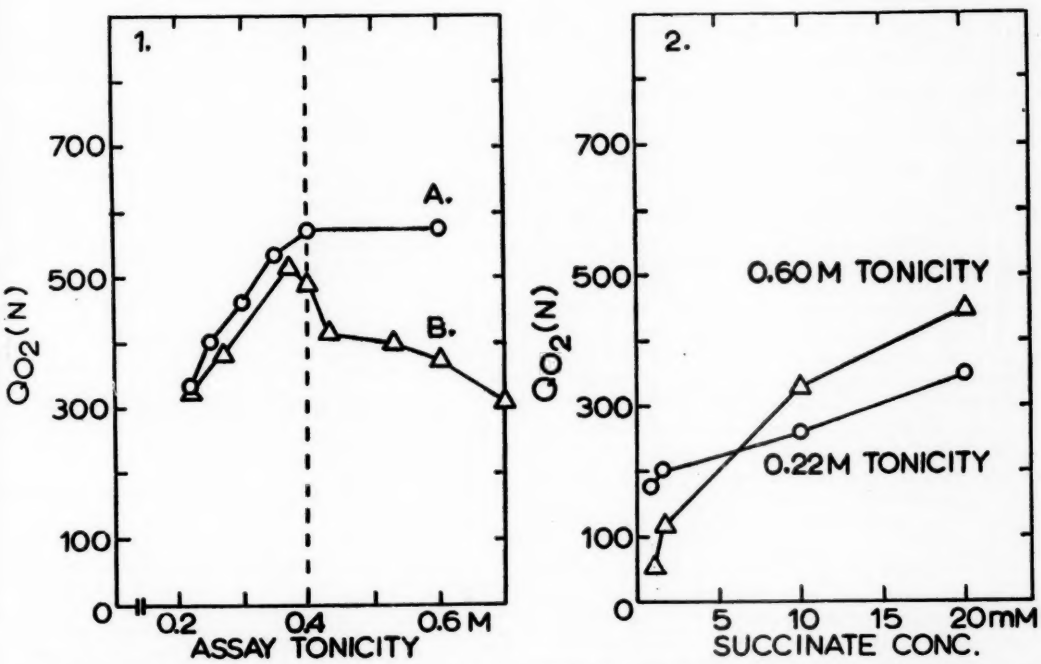

Fig. 1. Variation of succinoxidase activity with assay osmolarity. Mitochondria prepared in 0.74 M media and washed with 0.60 M sucrose. Assay media contained succinic acid (20 mM), tris (42 mM) Na_2ATP (601 μM), tris-acetate buffer (36 mM), and sucrose to adjust the osmolarity. Mitochondrial concentrations were 404 and 378 μg N/3 ml for A and B, respectively.

Fig. 2. Effect of assay osmolarity on the variation of succinoxidase activity with succinate concentration. Conditions as for figure 1 except for tris-succinate and assay osmolarity as indicated. Mitochondrial concentration was 477 μg N/3 ml.

TABLE I

OXIDATION OF SUCCINATE & MALATE BY LUPINE MITOCHONDRIA*, WITH & WITHOUT DPN

INITIAL CONCENTRATIONS			INITIAL Qo_2 (N)	O_2 UPTAKE/ mg N 60 min	R.Q. 60 min
SUCCINATE	MALATE	DPN			
20	0	0	344	266	0.0
20	0	0.5 mg/ml	380	253	0.1
16	4	0	290	206	0.0
16	4	0.5 mg/ml	274	177	0.0
0	10	0	0	9	1.2
0	10	0.5 mg/ml	0	19	0.8

* Mitochondria prepared in solutions of 0.60 M osmolarity transferred to 0.60 M solutions for gas exchange measurements at 30° C. Mitochondrial concentration was 596 μg N/3 ml. The assay media contained in addition tris (42 mM for 20 mM organic acid concentration, & 21 mM for 10 mM malate conc.), Na_2ATP (601 μM), magnesium acetate (33 μM), tris-acetate buffer (18 mM), and sucrose to adjust the osmolarity.

of succinate added in the mixture and the oxidation of malate was initially negligible. It seems likely, then, that the initial rates of oxidation measured upon succinate addition to lupine mitochondria can be attributed to succinoxidase activity.

FIRST ORDER KINETICS. Characterization of tonicity effects are shown in table II for relatively simple variations of experimental treatments in preparing the lupine mitochondria and in assaying for succinoxidase. Data are from selected experiments showing first order kinetics. General trends in changes of values considered more important than actual values which vary from experiment to experiment.

Table II shows that, regardless of the assay tonicity, progressive lowering of the osmolar concentration of the medium used for preparing the lupine mitochondria progressively decreased α. That is, malonate inhibition of succinoxidase changed from one with essentially competitive characteristics (α equals infinity) towards a non-competitive type (α equals unity). Low assay tonicity in comparison with high also decreased α except for mitochondria from 0.15 M preparations for which α was already near unity.

Lowering the osmolar concentration in the preparation increased both K_S and K_I (table II). Low assay osmolarity for succinoxidase from any preparation, however, decreased K_S. The ratio K_S/K_I was more consistently affected in that lowering either assay or preparation osmolarity decreased the ratio.

The data of table III were obtained by extending the assumptions and methods of Slater and Cleland (40) to the lupine succinoxidase system. Calculations of k_1', a composite rate constant which is also a function of substrate accessibility to enzyme (Slater & Cleland, 40), are attempts to characterize more closely the changes in K_S brought about by changes in tonicity. Since succinoxidase is a particulate multienzyme system and not a simple single enzyme, k_1' is only an apparent rate constant which is affected by the accessibility [i.e. permeability & diffusion of substrate to enzyme (cf. 40)]. It is assumed here that k_1' can be used to follow the effect of tonicity on accessibility.

Table III shows that enzymatic activity was unaffected by low assay tonicity except for mitochondria isolated in 0.60 M solutions. In the latter instance the activity was decreased 36 % relative to activity in the high assay tonicity. Succinate affinity and accessibility were increased in all cases by low assay tonicity. The changes in K_S brought about by assay tonicity largely can be attributed to changes in succinate accessibility except for mitochondria isolated in 0.60 M solutions. In the latter case only 60 % can

TABLE II

EFFECT OF OSMOLARITY ON APPARENT 1ST ORDER REACTION CONSTANTS FOR SUCCINOXIDASE ACTIVITY OF LUPINE MITOCHONDRIA

MEASURE	ASSAY* OSMOLARITY	PREPARATION OSMOLARITY**		
		0.15 M	0.40 M	0.60 M
K_S, mM succinate	0.22 M	5.10±1.42	2.87±0.50	1.20±0.10
	0.60 M	12.34±1.12	5.47±0.82	5.47±0.90
V_m, Qo_2 (N)	0.22 M	783±64	701±13	1099±44
	0.60 M	766±10	744±26	1491±24
K_I, mM malonate	0.22 M	0.91	0.19	0.05
	0.60 M	0.64	0.16	0.11
α	0.22 M	2	8	36
	0.60 M	1	191	268
K_S/K_I	0.22 M	5.6	15.1	24.4
	0.60 M	19.3	34.2	49.7
$\bar{r}$, coefficient of*** correlation, Lineweaver-Burk double reciprocal plot, malonate series	0.22 M	0.981	0.994	0.953
	0.60 M	0.879	0.994	0.977

* Activities measured in media of indicated osmolarities containing succinic acid (2–30 mM), tris (4–63 mM), Na_2ATP (601 μM), tris-acetate buffer (18 mM), tris-malonate (0.25 mM for 0.60 M preparations, otherwise 0.5 mM), magnesium acetate (50 μM for 0.60 M preparations, otherwise 33 μM), and sucrose to adjust osmolarities. Mitochondrial concentrations for the 0.15 M, 0.40 M and 0.60 M preparations were 376, 457, and 270 μg N/3 ml, respectively. Initial activities measured at 30° C.

** 0.40 M preparation assayed in 0.18 M and 0.57 M solutions.

*** All values significant at $P < 0.01$, except for $\bar{r}$ equals 0.879 which was significant at $P = 0.05$.

TABLE III

VARIATION OF EFFECT OF ASSAY OSMOLARITY ON ENZYMES OF SUCCINOXIDASE, ACCESSIBILITY OF SUCCINATE, & AFFINITIES OF SUCCINATE & MALONATE TO THEM*

MEASURE**	PREPARATION OSMOLARITY		
	0.15 M	0.40 M	0.60 M
Enzyme Activity Vm_A/Vm_B	1.02	0.94	0.64
Succinate Accessibility*** k'_{1_A}/k'_{1_B}	2.47	1.80	2.74
Succinate Affinity $(1/Ks_A)/(1/Ks_B)$	2.42	1.90	4.48
Malonate Affinity $(1/KI_A)/(1/KI_B)$	0.70	0.84	2.20

* Calculated from table II.

** Constants with subscript A determined in the 0.22 M assay and those with subscript B determined in the 0.60 M assay.

*** k'_1 is the composite rate constants which is also a function of the accessibility of succinate to the succinoxidase system. It is not the simple rate constant k′ since it not only concerns the concentration effect of reactants but also the diffusion of them to the sites of enzymic activity. k′ Was calculated according to Slater and Cleland whose assumptions for the cytochrome c oxidase system were extended here to the succinoxidase system (40).

be attributed to change in accessibility. In contrast to the increase in succinate affinity, malonate affinity was decreased by low assay tonicity, except for 0.60 M mitochondrial preparations whose malonate affinity increased. It is clear that the effect of tonicity in preparation and assay are different for malonate and succinate acting in the succinoxidase system.

SECOND ORDER KINETICS. In general lowering the preparation osmolarity decreased the effectiveness of both succinate and malonate in the succinoxidase system with first order kinetics (K_S & K_I increased, table II). This effect may also be related to the second order reaction, with respect to succinate concentration, shown by certain preparations. Most 0.15 M preparations of lupine mitochondria showed second order kinetics in assays of low osmolarities.

Table IV shows the effect of osmolarity and cytochrome c upon the apparent second order reaction constants of lupine succinoxidase. In common with the effect of preparation osmolarity on K_S/K_I in the first order reaction, K_1/K_2 was decreased progressively with the decrease in preparation osmolarity, where K_1 is analogous to K_S and K_2 to K_I. The apparent maximal velocity, v_m, was not affected in a regular way The effect of low tonicity was considered as possibly related to loss of a cofactor such as cytochrome c and the effect of added cytochrome c was studied. The effect of added cytochrome c was to increase v_m and K_1/K_2, except for 0.60 M preparations. That is, succinate seemingly became more readily bound in the apparent inhibitory position. However, it seems unlikely that mere loss of cytochrome c during the mitochondrial preparation in low osmolar solutions was involved because in the presence of added cytochrome c low assay osmolarity still decreased K_1/K_2 and v_m.

DISCUSSION

Our study on the effect of tonicity on succinoxidase demonstrates that reports of simple activation or inhibition are insufficient. Characterization of the tonicity effects by noting the change in the apparent reaction constants is less ambigious. The practice

TABLE IV

EFFECT OF OSMOLARITY & CYTOCHROME C ON APPARENT 2ND ORDER REACTION CONSTANTS OF LUPINE SUCCINOXIDASE

EXP.	PREPARATION OSMOLARITY	ASSAY OSMOLARITY	ASSAY* TREATMENT	K_1/K_2**	v_m** Qo_2 (N)
A	0.60 M	0.15 M	−Cyt c	1.31±0.02	982±48
			+Cyt c	1.16±0.16	1083±281
B	0.40 M	0.15 M	−Cyt c	0.073±0.001	779±15
			+Cyt c	0.386±0.001	1384±53
C	0.15 M	0.40 M	−Cyt c	0.125±0.003	642±59
			+Cyt c	0.379±0.012	1357±70
D	0.15 M	0.15 M	+Cyt c	0.034±0.003	832±46

* Assay media contained succinic acid (5–37.5 mM, Exp. A; 5–40 mM, Exp. B & C; 5–30 mM, Exp. D), tris (10–79 mM, Exp. A; 10–84 mM, Exp. B & C; 10–63 mM, Exp. D), Na_2ATP (601 μM), tris-acetate buffer (9 mM, Exp. A & B; 18 mM, Exp. C & D), and sucrose to adjust osmolarity. Cytochrome c where added was 0.20 μM. Mitochondrial concentrations were 434, 428, 467, and 336 μg N/3 ml for Exp. A, B, C, and D, respectively.

** K_1 and K_2 are the apparent dissociation constants for succinate in the activating and inhibiting positions, respectively, in the relation: $v_m/V = 1 + K_1/S + S/K_2$, where v_m is the apparent maximal activity and V the activity at S succinate concentration.

of correlating simple respiratory activity of mitochondria with physiological states in the intact cell is clearly based upon ambigious information if the order of reactions and the values of, at least, the apparent reaction constants are not determined.

In addition to variation of the values of the apparent first order reaction constants for lupine succinoxidase, we have obtained second order reactions with respect to succinate which may be related to the exposure of lupine mitochondria to solutions of low osmotic concentration. Data from earlier studies also showed inhibition by high succinate concentration of succinoxidase from rat liver mitochondria (38) and pigeon liver mitochondria (19). These data followed second order kinetics but the investigators did not comment upon it. It is significant that both the rat and pigeon liver mitochondria were subjected to hypotonic stress in preparation. The procedure developed for the rat liver mitochondria by Schneider and Potter (38) has been widely used.

Tonicity clearly affected lupine succinoxidase in two ways, at least, with the properties analogous to the affinity and accessibility of succinate to the system being affected relatively more than the properties influencing the overall enzymic rate. The pattern in the effect of lowering the preparation tonicity showed succinate becoming less and less effective as substrate (K_S increased) and, indeed, even becoming inhibitory as shown by second order reactions. However, succinate functioning as substrate became relatively more effective compared with the inhibitory action of malonate in the first order reaction and succinate as inhibitor in the second order reaction. It may be more helpful to regard the lowering of preparation tonicity as decreasing the effectiveness of both substrate and inhibitor, but the latter relatively more. This was shown by the decreases in the ratios of K_S/K_I and K_1/K_2.

Malonate has been used as the classical example of a competitive inhibitor (33). It is therefore surprising that values of α were obtained ranging from 270 to 1; that is, malonate changed from affecting primarily the affinity of succinate (competitive inhibition) to inhibiting the overall rate (non-competitive inhibition, α equals 1, cf.5). Departure of malonate inhibition from the pattern of competitive inhibition has not been previously reported. Examination of other published graphs, however, shows that some values of α, in fact, were different from infinity although high. Friedenwald and Maengwyn-Davies (10) noted that some inhibitions have been called competitive when α was as low as 10 and that when α was as large as 2 some inhibitors have been classified as non-competitive.

No quantitative relation between the osmotic swelling properties and succinoxidase activity of lupine mitochondria have been noted. However, mitochondria prepared in 0.15 M solutions usually showed second order reaction rates in the oxidation of succinate; and their optically-measured volumes (1/optical density of suspension) no longer varied inversely with osmolar concentration (16).

It is clear that transfer of lupine mitochondria from one solution to another differing in tonicity has marked effects upon the succinoxidase system. There is uncertainty about the relations between mitochondrial swelling, electron transfer in respiratory pathways, and the effect of various agents which influence phosphorylation and electron transfer (cf. 7). We may anticipate that in some cases insufficient characterization has contributed to confusion and that a more complete description by determination of even the apparent kinetics of the systems will help to provide rational and consistent explanations.

Summary

I. Progressive lowering of the osmolar concentration of solutions used both for isolating lupine mitochondria and enzyme assay progressively decreased α, a measure of the interaction of the malonate and succinate enzyme complexes in the first order reaction of succinoxidase, from values associated with competitive inhibition to values associated with non-competitive inhibition.

II. Both K_S and K_I, the apparent Michaelis constants for succinoxidase activation and inhibition by succinate and malonate, were progressively decreased by lowering the osmolar concentration used in preparing the mitochondria.

III. Low assay tonicity, compared with high, decreased K_S and progressive lowering of the preparation tonicity decreased the ratio K_S/K_I without affecting V_m in a parallel fashion.

IV. The apparent accessibility of succinate to succinoxidase was more affected by assay tonicity than was succinoxidase activity. Malonate affinity was not affected by assay tonicity in a parallel manner.

V. Lowering the preparation and assay tonicities also affected the order of succinoxidase activity with respect to succinate concentration. Most mitochondria isolated in 0.15 M solutions showed second order succinoxidase activity while those isolated in higher osmotic concentrations less frequently showed the second order reactions.

VI. For those preparations showing the second order reaction, lowering preparation tonicity decreased the ratio K_1/K_2, where K_1 and K_2 are the apparent dissociation constants for succinate in the activating and inhibiting positions, respectively.

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MECHANISMS BY WHICH WIND INFLUENCES TRANSPIRATION[1]

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Although the amount of transpiration from a leaf is predominantly a function of the amount of energy received by the leaf, wind can influence the manner in which the leaf loses energy, and thus can affect transpiration significantly. Wind influences transpiration by removal of the so-called "layer" of saturated air from the surface of the leaf, and also by changing the temperature of the leaf. Bange (1) showed that in the isothermal case the stimulation of transpiration by wind could be accounted for quantitatively by theoretical calculations involving the removal of the layer of saturated air. Thermal effects of wind on transpiration are more difficult to evaluate, since any change in transpiration rate will usually be accompanied by a secondary temperature change, but wind does have one direct effect on the leaf temperature. The increased mass of air brought into contact with the leaf by wind tends to bring the leaf temperature closer to the air temperature, regardless of whether the leaf be warmer or colder than the air.

Certain other mechanisms of wind action appear to be possible or have been mentioned in the literature as possibilities, and therefore deserve investigation. These mechanisms are:

I. Decrease in air pressure on the lee side of the leaf, causing increased evaporation on this side.

II. Ventilation of intercellular spaces, caused by actual passage of air through amphistomatous leaves.

III. Bending of the leaves in the wind, causing compression of the intercellular spaces and consequent pumping of saturated air out of the stomata.

These three mechanisms are the subject of this paper.

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I. DECREASE IN AIR PRESSURE. Maximov (5) mentioned that wind may cause a reduced pressure on the lee side of a leaf, causing increased evaporation on that side, and Gaumann and Jaag (4) invoke this same mechanism to account for part of their substomatal transpiration. Putting aside the fact that a reduced pressure on one side of the leaf would usually be compensated for by an increased pressure on the other, we can estimate the possible magnitude of this effect by considering a leaf held normal to a wind which is blowing 7 meters second^{-1} (ca. 15 miles/hr). Such a wind could cause a maximum pressure differential of about 570 dynes cm^{-2} between the two sides of the leaf. This 570 dynes cm^{-2} would be less than 0.06 % of the difference between atmospheric pressure and the vapor pressure of water. Therefore the rate of transpiration could not be increased by more than 0.1 % by such a pressure differential.

II. VENTILATION THROUGH AMPHISTOMATOUS LEAVES. Measurements made by a modification of the Darwin and Pertz (2) porometer technique indicate that a pressure of 10^4 dynes cm^{-2} may force a maximum of 10^{-3} cm^3 of air per second through 1 cm^2 of a corn leaf when the stomata are open. Thus a 7 m second^{-1} wind, causing a pressure differential of 570 dynes cm^{-2} between the sides of the leaf, might force as much as 5.7×10^{-5} cm^3 of air per second through each square centimeter of a corn leaf.

Miller and Coffman (9) found that corn plants in the field often transpired as much as 5.5×10^{-6} g of water per square cm of leaf per second (200 g m^{-2} hr^{-1}). If the relative humidity inside the leaf were 100 % and that outside of the leaf were 50 %, an exchange of 3.6×10^{-1} cm^3 of air per cm^2 of leaf sur-

face would be required each second to account for this transpiration. The air exchange forced by a 7 m second^{-1} wind, then would be sufficient to account for only 0.016 % of the observed transpiration.

III. Pumping by Leaf Bending. Both the older and the recent literature (6, 7, 8, 10) have numerous references to the possibility that the change of volume of intercellular space as leaves bend in the wind might pump gases out of the stomata and thus increase transpiration. The only experimental work bearing upon this pumping action seems to be that of Eberdt (3), who found that leaves which were allowed to flutter in moving air lost about 10 % more water than did fixed leaves. Unfortunately, Eberdt's description and data permit neither an evaluation of possible artifacts nor a statistical analysis.

Leaves actually undergo very little bending in the wind. The maximum rate of bending of corn leaves, for instance, seems to occur at wind speeds of 7 to 10 meters second^{-1}. Stronger winds break and tear the leaves. There are two types of leaf motion—a slow flexing of most leaves with a period of about two seconds and a radius of curvature changing between limits such as 30 cm and 50 cm, and a rapid flutter of a few leaf tips with a period of as little as 0.25 seconds and a radius of curvature of 3 cm or more.

With a 3-cm radius of curvature, the stretching of the outer surface or the compression of the inner surface of a corn leaf 0.018 cm thick would be 0.6 %. If all of this stretching or compression took place in the intercellular space, the maximum possible volume change would be 10^{-4} cm^3 of air per cm^2 of leaf. At four cycles per second, 4×10^{-4} cm^3 of air would be exchanged each second. This would be only 1/9th of 1 % of the 3.6×10^{-1} cm^3 cm^{-2} second^{-1} of air exchange necessary for the transpiration rate observed by Miller and Coffman. Thus the pumping action of leaf bending theoretically could not account for a significant portion of the transpiration.

Experimental

Sections from the tip portions of corn leaves were harvested in the field and kept in polyethylene bags until they were used. These sections were 16 cm long, and the average widths were 6.3 cm and 8.5 cm at the tip and basal ends, respectively. Two leaf sections, designated A and B, were fastened by rubber bands to leaf holders, one of which is shown diagrammatically as figure 1. A fan and a lamp were arranged to give a wind velocity of 5 m second^{-1} and a light intensity of 40 lux on both leaf sections. Temperature was not controlled, and ranged between 28° C and 32° C. A motor was arranged so as to flex each leaf. Four 3-minute runs were made with each pair of leaf sections. In the first and third runs leaf section A was the "fast" leaf, flexing at 4 cycles per second and leaf section B was the "slow" leaf, flexing at one cycle per second. In the second and fourth runs, A was the slow leaf and B was the fast leaf. Leaf

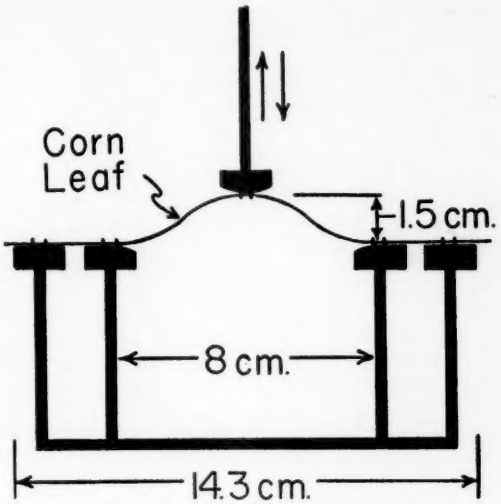

Fig. 1. Leaf on leaf holder (diagrammatic).

weights were taken initially and after each run, and the data were analyzed statistically to determine if there was a significant difference between the amount of water lost by the fast leaves and that lost by the slow leaves. Theoretical calculations indicated that the fast leaves should transpire no more than 0.13 % faster than the slow leaves. A similar experiment was conducted without a fan, as a test of the method.

Results & Conclusions

In 60 individual runs, the fast leaves lost 0.33 % more water than did the slow leaves, but this difference was far from being statistically significant. The variability was such that the least significant difference would have been 3 %, so we can say with some degree of assurance only that the fast flexing would not increase the transpiration by as much as 3 % under these conditions.

In 32 individual runs without the fan the fast leaves lost 6.2 % more water than did the slow leaves. This difference was statistically highly significant. Such a difference would be expected even in the absence of a pumping action, because the flexing would stir the air in the vicinity of the leaf.

Summary

The following three mechanisms by which wind might possibly increase transpiration have been explored theoretically:

I. Increase in transpiration caused by a decrease in air pressure on the lee side of the leaf.

II. Ventilation of amphistomatous leaves by air passing through the leaf by way of the stomata and intercellular spaces.

III. Pumping action of changes in volume of intercellular spaces as leaves bend in the wind.

It is concluded from theoretical considerations that none of these mechanisms could account for as much as 1 % of the transpiration of corn leaves.

The pumping action of leaf bending was also investigated experimentally by means of an apparatus which flexed leaves in a stream of air. The flexing did not cause as much as a 3 % difference in the transpiration.

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ACTION SPECTRA OF LIGHT-SATURATED PHOTOSYNTHESIS[1]

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At low light intensities the rate of photosynthesis is directly determined by the intensity and color of the light absorbed. It is only slightly influenced by temperature and carbon dioxide concentration.

At high light intensities a saturation is reached so that a further increase in the light intensity does not increase the rate of photosynthesis. Typical curves of photosynthetic rate versus intensity are given by Rabinowitch (12). The photosynthetic rate at saturating intensities is determined by the temperature, carbon dioxide concentration, and the physiological state of the plant. It has generally been supposed to be independent of the wavelength of the light.

This paper deals with the photosynthetic rate at different wavelengths above the level of light saturation, but below intensities causing photochemical damage to the plant. Contrary to expectation there was structure in the action spectrum of photosynthesis at saturating light intensities.

Methods

The polarographic apparatus used for measuring the rate of oxygen evolution by photosynthesis was patterned after Haxo and Blinks (7) as modified by Haxo and Fork (8) for use with suspensions of unicellular algae.

A 1,000 w General Electric projection lamp, and a high-pressure water-cooled mercury lamp (PEK Labs, Palo Alto, Cal.) served as sources of light. An image of each lamp was focused simultaneously on the platinum electrode by large condensor lenses and mirrors. Adjustment of the light intensity was made with variable voltage transformers. Interference filters were combined with colored glass filters to give reasonably monochromatic light (ca. 10 mμ half band-width). Infrared radiation was removed with water and "Calflex" infrared reflecting filters.

The unicellular algae, *Chlorella pyrenoidosa* Chick (a chlorophyte), *Phormidium persicinum* (Reinke) Gomont (a cyanophyte), and the chrysophyte *Botrydiopsis alpina* Visher were used. Chlorella was cultured in Knop's solution in a continuous culture apparatus (9); Phormidium was cultured in a modified sea water medium (11) under daylight illumination and, Botrydiopsis was cultured on Bristol's agar medium under continuous illumination from two 15 w fluorescent tubes.

Cells from the culture of Chlorella were centrifuged and diluted with their respective media to 25 cmm/cc. This suspension was pipetted onto the platinum electrode at the bottom of a well 0.25 mm deep, and covered with a piece of moistened dialyzing

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membrane. Cells of Botrydiopsis were scraped from the agar and spread in a thin layer as a paste on a platinum electrode set flush with the adjacent supporting surface of lucite. Phormidium was studied by lifting mats of filaments which had grown out very uniformly at the surface of sea water and spreading them as smoothly as possible over the flush electrode.

After introducing the algae the whole assembly was immersed in 650 cc of the growth medium which had been aerated with 5 % carbon dioxide in air. During the measurements 5 % carbon dioxide in air flowed through the gas phase of the electrode assembly. The temperature was held constant at 21 to 22° C.

The light intensity on the cells was tested for saturation by a wire screen which transmitted 68 % of the incident light. If, when the screen was removed, there was less than a 1 % deviation in the rate of photosynthesis, the algae were judged to be adequately light saturated for that wavelength.

Results

Chlorella, an alga with chlorophyll a, the carotenoid accessory pigments, and chlorophyll b, was used for many of the experiments of photosynthesis at saturating light intensities. At the start of the record, a dark period was followed by a period in the light at a selected wavelength. To be certain that a maximal saturation rate had been achieved, the algae were given a 15 minute exposure to light. When the screen was put between the light source and the algae no deviation of the photosynthetic rate was recorded showing that the algae were at a saturation level for this wavelength. After another dark period the light was turned on and a level of saturation measured for light of another wavelength.

A comparison of the differences in the recorder deflection during exposure to saturating light and in darkness, gave the high intensity action spectrum of Chlorella (fig 1). To include the many sets of

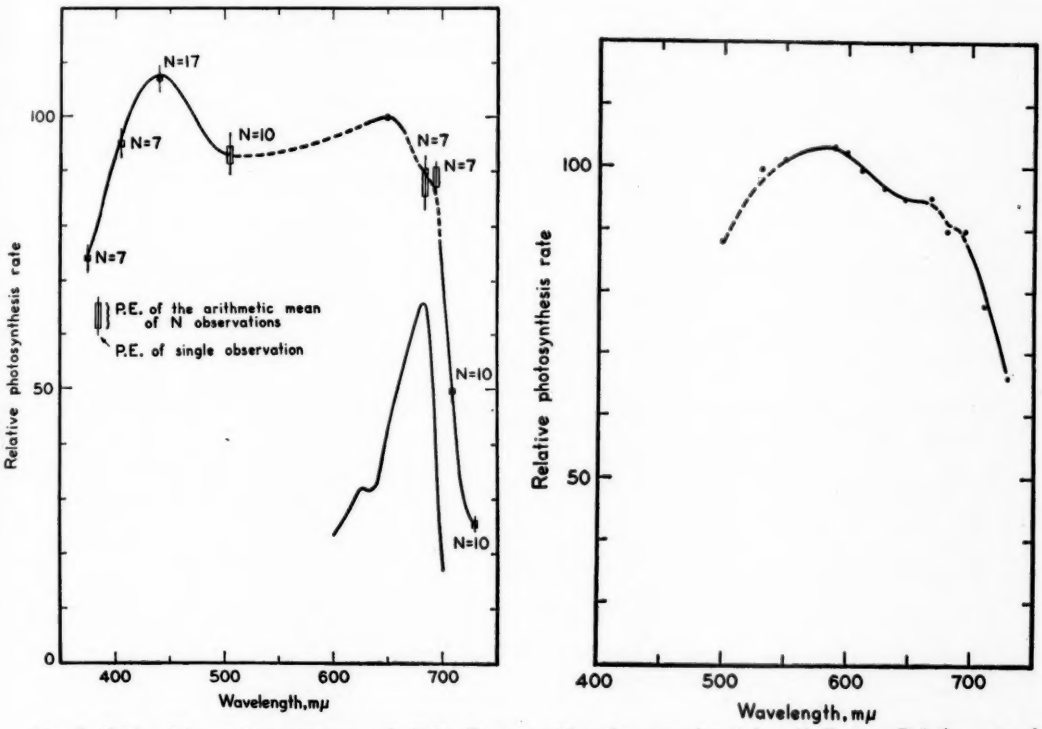

Fig. 1 (*left*). The action spectrum of *Chlorella pyrenoidosa* in saturating light. Ordinate: Relative rate of photosynthesis: abscissa, wavelength. The insert of figure 1 shows the low intensity action spectrum of photosynthesis in Chlorella. The formulas used to calculate the statistics are: A: The probable error of a single observation = $0.6745 \times \sqrt{\frac{\Sigma(v^2)}{n-1}}$ B: The probable error of the arithmetic mean of N observations = $0.6745 \times \sqrt{\frac{\Sigma(v^2)}{n(n-1)}}$

Fig. 2 (*right*). The action spectrum of *Phormidium persicinum* in saturating light. Ordinate: Relative rate of photosynthesis; abscissa, wavelength.

measurements the data were normalized at 650 mμ. The vertical line at each wavelength is an estimate of the probable error of a single observation whereas the rectangle is an estimate of the probable error of the mean for the number of observations, "N".

The resulting action spectrum has maximum at 440 mμ. The rate of photosynthesis at saturating intensities of 710 mμ and 376 mμ is about half that at the peak position; and, photosynthesis when saturated at 730 mμ is one-quarter that of the peak value. There is a minimum in the spectrum between 650 and 510 mμ and a suggestion of either a maximum or a shoulder in the wavelength regions of 650 mμ and 680 to 694 mμ.

When photosynthesis in Chlorella was measured at a saturating intensity of 650 mμ the addition of saturating intensities of 710 mμ gave no increase of rate.

Figure 1 (insert) also shows the low intensity action spectrum of photosynthesis in Chlorella as measured with the automatic recording arrangement of French, Mayers, and McLeod (6). The low intensity curve is the reciprocal of the relative number of incident quanta required to keep the photosynthetic rate constant as the wavelength varies. The actual rate used for the low intensity curve was about 0.006 that of the rate at saturation at 650 mμ. A different culture of the same strain was used on the same electrode as was used for the high intensity spectra. The rate scales are not comparable and the low intensity curve is greatly enlarged.

High intensity action spectra for algae containing chlorophyll a, accessory carotenoid pigments, and phycobilins, but no chlorophyll b were studied between 483 and 730 mμ. The action spectrum for Phormidium shows a maximum in the region of phycocyanin absorption (550 mμ–615 mμ), and a shoulder in the region of chlorophyll absorption (fig 2).

The action spectrum for Botrydiopsis, an alga that has chlorophyll a, accessory carotenoid pigments, but no chlorophyll b, is shown in figure 3. Measurements with and without an intervening dark period agreed and are both included in the figure. In this alga, the spectrum resembles that of Chlorella except that the maximum in the red is at 695 mμ rather than at 650 mμ, and the blue maximum is at 420 mμ rather than at 440 mμ. There is also an indication of a third peak at about 600 mμ.

The results show, however, that in saturating light the rate of photosynthesis is dependent upon wavelength. This would not have been expected from the classical explanation of the shape of the curve relating photosynthetic rate to light intensity.

The Emerson effect has been defined as the increased rate of photosynthesis obtained by illumination with two wavelengths simultaneously as compared to the sum of the photosynthetic rates produced by each separately. Emerson (2) discovered that light at about 700 mμ, which is usually inefficient for the photosynthesis of green plants, becomes more efficient when shorter wavelengths are given simultaneously. The action spectrum of the enhancement effect caused by shorter wavelengths as measured by Emerson (3) and by Myers and French (10) was interpreted as showing that chlorophyll b was the main pigment whose activation increased the effectiveness of long wavelength red light. Furthermore, Emerson (4) and also Blinks (1) found from action spectra for enhancement that in algae with accessory pigments, but lacking chlorophyll b, the phycobilins act similarly to chlorophyll b in Chlorella for causing enhancement of photosynthesis at long wavelengths. The peak at 650 mμ in the action spectrum of Chlorella at saturating light intensities suggests that the Emerson enhancement effect and the structure shown in the high intensity action spectrum may have a common origin.

In Botrydiopsis, lacking chlorophyll b and other accessory pigments, the maximum in the high intensity action spectrum appears at 695 mμ rather than at 650 mμ as in Chlorella. The action spectrum of Botrydiopsis at low light intensity has the peak at 680 mμ. At low light intensity this alga shows enhancement of photosynthesis from the wavelength pairs 670 mμ and 695 mμ. Presumably the enhancement is due to increased effectiveness of one form of chlorophyll a (5).

The enhancement of photosynthesis at low light intensities has been found to occur when the 695 mμ form of chlorophyll a and an accessory pigment such as chlorophyll b, or a phycobilin absorbs light. The action spectrum at high intensity approximates the action spectrum for the enhancement. As a consequence the high intensity action spectrum here reported may be related to the enhancement effect but the precise way in which this comes about is far from clear at the present time.

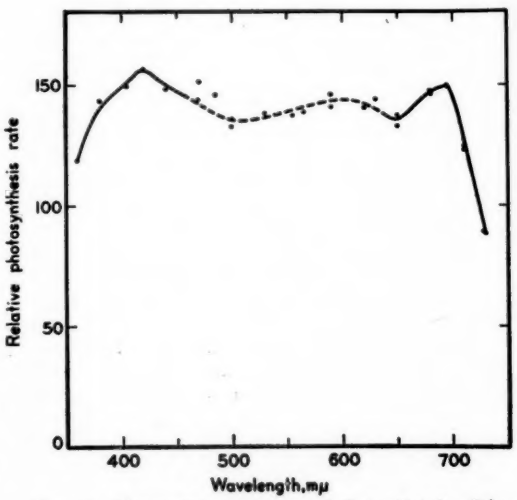

FIG. 3. The action spectrum of *Botrydiopsis alpina* in saturating light.

Summary

The action spectrum of photosynthesis at saturating light intensities was investigated.

The results show that in saturating light the rate of photosynthesis is dependent upon the wavelength of the incident light.

The Emerson enhancement effect may be responsible for the structure of the high intensity action spectrum.

Acknowledgment

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CYANIDE & CHELATING AGENT EFFECTS ON IN VITRO CO_2 FIXATION IN SWEET ORANGE LEAVES[1, 2]

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Heavy metals inhibit and chelating agents often stimulate CO_2 fixing reactions (4, 5, 6). Cyanide, which combines with and inactivates many metals, was used in the present CO_2 fixation studies to separate the effect of chelating agents on heavy metals from other possible effects of chelating agents. Cyanide and chelating agents have been successfully used to overcome heavy metal inactivation of certain enzyme-catalyzed reactions (1, 7). An example of an additional effect of chelating agents on biological reactions was indicated by Yang (9) who reported that a chelating agent increased respiration rate and that the effect was independent of overcoming inhibiting effects of excess cations. Similar effects have been looked for with systems fixating $C^{14}O_2$. The ability of cyanide to combine with aldehydes and ketones (8) was also of interest in these studies as a method of stabilizing reaction products.

MATERIALS & METHODS

For all the studies the source of enzymes was preparations from sweet orange (*Citrus sinensis* (Linn.) var. Blackman Sweet Orange) leaves from a tree in the orchard at U.C.L.A. One gram of leaves was ground with 6 ml 0.2 M, pH 8.0 tris(hydroxymethyl) aminomethane (tris) buffer with a mortar and pestle at 0° C. The material was otherwise prepared for assay as previously described (4).

Phosphoenolpyruvate (PEP) and ribose-5-phosphate (R5P) were used as substrates as previously described (4, 5, 6). Stock solutions of 0.05 M NaCN, 0.05 M NaN_3, and 0.01 M ethylenediaminetetra acetate (EDTA) were prepared at pH 8.0.

The methods for assay of CO_2 fixation were as described previously (5). Briefly, substrates and cofactors were incubated with 5 μM $HC^{14}O_3^-$ containing 1.2 $\times$ 10^6 CPM as determined as the $BaCO_3$ precipitate with a thin window Q-gas counter and with 0.1 ml of the homogenate preparation in a 1.0 ml volume for 10 minutes in a metabolic shaker at 37° C. The reaction was stopped by adding 0.1 ml of concentrated HCl and the tube was immediately evacuated through a KOH trap. The samples were centrifuged to remove precipitated proteins. Aliquots of 0.2 ml were dried in pyrex planchets with forced air at room temperature before counting with a thin window Q-gas counter. All experiments were repeated a minimum of four times.

RESULTS & DISCUSSION

The data in table I show the effects of cyanide on the two carboxylating systems when added directly to the reaction systems and also when added at the conclusion of the incubation period so that cyanide was present during the drying period only (see table I). Cyanide greatly inhibited the R5P system supposedly because of cyanohydrin formation with ribulose-5-phosphate and with ribulose-1,5-diphosphate (8). Cyanide stimulated the phosphoenolpyruvate (PEP) carboxylase catalyzed reaction or at least resulted in an increased C^{14} fixation. For both reactions adding cyanide at the conclusion of the incubation period resulted in somewhat higher C^{14} activity in the planchets. In general the effect was more pronounced for the PEP reaction than for the R5P. The cyanide had to be added before the acid rather than after to have an effect. Separate studies showed that the effect of cyanide was not related to pH changes.

TABLE I

EFFECT OF DIFFERENT LEVELS OF NaCN ON R5P & PEP REACTION SYSTEMS

TIME OF NaCN ADDITION	μM NaCN 0	1	5	10
	CPM/ALIQUOT			
	R5P			
During reaction	9,800	6,700	640	280
Before drying*	9,800	11,000	12,000	11,000
	PEP			
During reaction	2,400	2,700	3,900	4,200
Before drying*	2,400	2,600	2,900	3,100

* Before drying in this case was before the acid was added to the reaction mixture.

Possibilities for explanation of the in vitro effect on the PEP system include inactivation of toxic heavy metals, combination with and stabilization of labile intermediates and products, enzyme activation, and inactivation of competing reactions.

[1] Received revised manuscript October 20, 1960.

[2] This study was supported in part by the United States Atomic Energy Commission, Contract AT(11-1)-34, Project no. 51.

TABLE II

EFFECT OF CYANIDE LEVEL ON OVERCOMING IRON INHIBITION OF PEP CARBOXYLASE REACTION

NaCN μM	CPM/ALIQUOT
0–minus Fe*	2,500
0	1,900
1	2,000
2	2,500
5	2,700
10	3,500

* All other samples received 1 μM $FeSO_4$.

The first possibility was tested in a series of studies. In table II are data indicating that cyanide when added to the PEP system not only overcame the inhibitory effect of Fe^{++} addition to the system but also increased the activity above the base reaction.

Table III shows that cyanide overcame the inhibition at least in part of a number of heavy metals with the exception of zinc. A combination of zinc and cyanide during the reaction consistently caused greater inhibition. When zinc was added during drying only the effect was different. It was noted that the depressing effect on C^{14} activity in planchets caused by some metals could be overcome in part by adding the cyanide after the incubation and before the drying step. One metal effect was expressed on the enzyme-catalyzed reaction and another effect was expressed on the stability of reaction products.

To further test whether or not the main effect of the cyanide was in prevention of heavy-metal-induced decarboxylation of reaction products during the drying process, heavy metals were added to planchets after the aliquots of the reaction mixtures. Fe, Zn, and Hg at 10^{-3} M levels resulted in very little decarboxylation of the fixed C^{14}-products.

As a further test on heavy metal effect, 80 % saturated ammonium sulfate precipitates were made, taken up in 0.2 M tris buffer with 0.01 M EDTA and dialyzed free of ammonium sulfate and EDTA in the presence of 0.01 M tris. This was done to remove at least some of the contaminating metals and possibly decrease the cyanide effects. The cyanide and chelate effects, however, were still present (table IV). The experiment was repeated with EDTA also in the precipitating solution to further remove heavy metals. The results were similar.

TABLE III

EFFECT OF CYANIDE ON INHIBITOR EFFECTS OF HEAVY METALS ON PEP CARBOXYLASE REACTION

HEAVY METAL	CONCENTRATIONS M	NaCN ADDITION (5×10^{-3} M) NONE	DURING INCUBATION	AFTER INCUBATION & BEFORE ADDING ACID
		CPM/Aliquot		
Control	...	2,800	5,500	3,800
$FeSO_4$	2×10^{-3}	1,300	3,800	3,000
$HgCl_2$	2×10^{-5}	1,700	4,900	2,100
$ZnCl_2$	5×10^{-4}	2,300	1,200	2,800
$CuSo_4$	10^{-3}	1,600	2,500	2,000
$MnSo_4$	10^{-3}	2,000	3,900	2,400

The formation of the hydrazone of 2,4-dinitrophenylhydrazine (DNPH) and oxalacetic acid (OAA) is a common technique for stabilization of the OAA when dried in planchets as used in this study. Since cyanide has a similar effect, the possibility of additive effects between the DNPH and the cyanide was investigated. The two reagents had the same effect when added during drying in that they were not additive. The DNPH appeared to effectively stabilize the OAA in that the results showed that cyanide and DNPH both resulted in identical increases when added after the incubation. The two chemicals also were not additive when the cyanide was added to the reaction system. A considerable amount of the cyanide effect on the PEP reaction system, therefore, was in stabilization of the OAA as it was synthesized. This actually could move the reaction to the right because of a mass action effect.

TABLE IV

EFFECT OF NaCN ON R5P & PEP REACTION SYSTEMS WITH AMMONIUM SULFATE-PRECIPITATED, EDTA-PURIFIED, & DIALYZED HOMOGENATES

TREATMENTS	PEP CPM/ALIQUOT	R5P CPM/ALIQUOT
Control	2,800	850
2 μM EDTA	3,300	4,100
10 μM NaCN in incubation	8,900	160
10 μM NaCN before acid	5,100	1,000
EDTA & NaCN before acid*	4,900	4,700

* The EDTA (2 μM) was present during the incubation and the NaCN added at the conclusion of the incubation and before the acid.

By using $KC^{14}N$ and standard OAA the cyanohydrin of OAA was demonstrated in the reaction mixtures. With 1 μM OAA per 1.0 ml of reaction mixture and 10 μM of $KC^{14}N$, essentially all of the OAA was in the cyanohydrin form. With 10 μM OAA, about 50 % of the OAA was as the cyanohydrin. By silica gel fractionation the cyanohydrin of OAA was separated from the products of the PEP carboxylase catalyzed reaction. This was a large single peak in fractions 100 to 130 in the separation procedure of De Kock and Morrison (3). This peak was not observed in the R5P reaction system.

Bradbeer et al (2) have shown that $C^{14}O_2$ fixation via the carboxylation enzyme results in synthesis

of some OAA. The small cyanide effect observed on the R5P products during the drying procedure may also be the formation of the cyanohydrin of OAA but this was not proved.

Azide produced effects somewhat similar to cyanide. This is illustrated in table V for a system partially cleaned and purified as before. The azide resulted in increased activity with both the R5P and PEP systems. Its effect was less pronounced than with cyanide on the PEP system and may be, in contrast to cyanide, an effect on heavy metals only. A reason for the studies represented by table V was to compare the effect of a chelating agent with cyanide and azide. The effects were additive. This indicates the possibility that the stimulating effect of chelating agents on the two carboxylating systems was other than primarily overcoming heavy metal inhibition.

TABLE V

ADDITIVE NATURE OF CHELATE, CYANIDE, & AZIDE EFFECTS ON $C^{14}O_2$ FIXATION WITH PEP & R5P AS SUBSTRATES IN PREPARATIONS FROM AMMONIUM SULFATE PRECIPITATED & DIALYZED SYSTEMS

μM EDTA	PEP			R5P	
	CONTROL	NaCN	NaN_3	CONTROL	NaN_3
0	2,000*	4,500	2,400	3,600	5,500
1	2,300	5,300	2,700	6,700	8,500
5	2,500	5,800	2,900	7,900	9,100

* CPM/Aliquot.

SUMMARY

Cyanide, azide, and chelating agent effects on in vitro dark $C^{14}O_2$ fixation studies with R5P and PEP as substrates with preparations from sweet orange leaves were compared. Cyanide increased the fixation when added with the PEP during the incubation period. When added just before the acid that was used to stop reactions and expel unreacted bicarbonate, cyanide resulted in increased C^{14} counts on planchets for both substrates but the effect was much less than when present during the incubation. The major effect of cyanide appeared to be in stabilization of OAA by formation of a cyanohydrin. The OAA cyanohydrin was identified when the PEP substrate was used. Removing heavy metals did not alter the increased fixation resulting from cyanide. Cyanide overcame inhibitory effects of heavy metals when the latter were added to the PEP reaction system except for zinc. In general the metal effects were on the reactions themselves rather than on metal-induced decarboxylation of products. The stimulating effect of a chelating agent was additive with either cyanide or azide and occurred in a system cleaned of heavy metals by ammonium sulfate precipitation with EDTA and dialysis.

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AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Executive Committee Meeting, Oklahoma State University, Stillwater, Oklahoma, 28 August 1960*: The Executive Committee Meeting was called to order at 8:30 pm by President Burris. Those present were: H. Beevers, W. D. Bonner, A. H. Brown, R. H. Burris, W. C. Hall, S. B. Hendricks, R. W. Kraus, C. L. Leinweber, B. S. Meyer, C. O. Miller, A. W. Naylor, G. R. Noggle, F. K. Skoog, and B. Stowe.

The minutes of the previous meeting were approved as printed in Plant Physiology.

The executive secretary-treasurer, Dr. G. R. Noggle, presented his annual report. During the year the membership climbed to 1,616, and the net worth decreased from $89,437.54 to $87,217.98. Dr. Noggle's report and budget of $3,100 were unanimously approved. In addition, the president was instructed to authorize an additional $500 for operating expenses of the executive secretary-treasurer if both the president and the executive secretary-treasurer feel it is necessary. Some details of Dr. Noggle's report follow:

MEMBERSHIP TOTALS

	July 1, 1959	July 1, 1960
Life members	35	35
Corresponding members	19	17
Foreign members	215	216
U. S. members—Regular	1,106	1,175
Student	140	178
	1,515	1,621
Less: those included in other groups	5	5
TOTAL	1,510	1,616

SUBSCRIPTIONS

U. S. subscriptions	V. 34	459	V. 35	517
Foreign subscriptions	V. 34	953	V. 35	977
TOTAL		1,412		1,494

SUMMARY OF CASH ACCOUNTS

Checking account, July 1, 1959	$ 22,660.63
Savings account, July 1, 1959	17,946.75
	$ 40,607.38
Receipts 1959-60	37,169.85
TOTAL	$ 77,777.23
Expenditures, 1959-60	40,346.75
CASH BALANCE, July 1, 1960	$ 37,430.48
Cash on hand, July 1, 1960	
Checking account	$ 18,895.20
Reserve account	18,535.28
TOTAL	$ 37,430.48

Dr. A. H. Brown, editor of Plant Physiology, presented a report on activities of his office for the past year. Dr. Brown also proposed for the coming year a budget of $4,800 to cover office expenses and $19,500 to pay for printing and mailing costs. The editor's budget and report were approved by all people present. The substance of the editor's report follows.

There is a cliche of travelers to remote lands which defines a successful expedition as one without adventures. The underlying truth of the saying applies to many endeavors and the editor may be permitted to apply the thought even to the humdrum activities of an editorial office: an uneventful year ought to be a satisfactory one for the Journal—the more humdrum, the better. In this sense the past year was so "successful" that the annual report of the editor ought to seem dull indeed. Nevertheless some things should be reported even if only because it has become traditional to do so.

STYLE CHANGES: The American Institute of Biological Sciences has just issued a *Style Manual for Biological Journals*. This was prepared by the Conference of Biological Editors—AIBS, Committee on Form and Style under the chairmanship of Dr. J. R. Porter. Two members of the ASPP served on this ten-man committee. The Journal was kindly provided with a preprint of the Style Manual so that our Instructions for Contributors (Jan. 1960 issue) complies with it. In the July 1960 issue we began the slow process of introducing style changes to conform with the Manual. It was nearly impossible to introduce all changes at one time; each subsequent issue carried additional changes. Beginning with the January 1961 issue all changes will essentially have been introduced. Our most noticeable policy revision pertains to the form of literature citations. Equally important are standardized symbols and abbreviations. Other changes, relatively minor, will generally tighten our editorial handling of style (which does not usually arouse the active interest of authors but which concerns conscientious editorial assistants with an urge to improve journalistic standards and an understandable fetish for consistency.

EDITORIAL OFFICE POOL: The AIBS has embarked on an interesting venture calculated to lighten the burden of paperwork which many professional societies provide to occupy the "spare" time of business officers and editors. A pool of business and editorial offices has been set up between professional societies and the AIBS to contract for the purely mechanical aspects of running a journal or a society. Either the routine paper work of the executive secretary-treasurer, or the processing of accepted manuscripts, or both can be handled by the AIBS for a fee. On the assumption that a large consolidated operation is likely to be more efficient than a small one, it might seem that the AIBS redactorial service could publish our Journal for less than our present cost. Furthermore, any routine work which could be removed from the editor's shoulders would restore just that much of his presumably valuable time for pedagogic or more scientifically challenging activities.

As far as PLANT PHYSIOLOGY is concerned neither argument seems to hold water. It would cost approximately $2.75 per page for the AIBS to handle all mechanical phases of publishing our Journal. At present this costs us slightly more than $2.00 per page. This does not necessarily mean that the AIBS cost estimate is unrealistic. It means that the Journal benefits by not having to pay for such hidden costs as office space, janitorial service, use and repair of office machines, and other overhead. We are happy to let such costs remain hidden.

The argument that the editor's work load would be reduced by the AIBS redactorial service seems not to be correct as long as the editor retains all his *essential* functions: selecting reviewers, evaluating reviews and manuscripts, and making other editorial decisions. He also must prepare an annual report to the Editorial Board. These activities occupy well over 90 % of the editor's Journal time. The purely mechanical work of the editorial office is delegated to his parttime editorial assistant, Mrs. Sukalo, and to a parttime clerk-typist. To remove entirely all copy editing and routine processing of accepted manuscripts from the editorial office could lighten the editor's work by perhaps 5 or 10 %. A clerk-typist still would be necessary and it is conceivable that it would require more time for the editor to handle correspondence with the redactory service. While it would be immodest for the editor to suggest that there is no room for improvement it does seem that the work of the editorial office is appropriately distributed in accordance with the talents of the personnel involved.

By arranging for the AIBS to provide professional redactorial service and by reducing our office staff we should not expect to realize any material advantage. As a result the editor recommended that we continue to operate just as we do now.—at least for the duration of his term of office.

IMPROVED REPRODUCTION METHODS: Trends in science being what they are it does not surprise us to find microscopists publishing some excellent work in what can only be referred to broadly as plant physiology. This is inevitable because the potential of the electron microscope is being exploited in so many research areas. In the past our Journal has received few manuscripts containing electron photomicrographs (in spite of the general suitability of the subject matter for PLANT PHYSIOLOGY.) Authors of such papers have been exceptionally critical and usually insist on reproductions of the highest quality. To an outsider it almost seems as if it has become a fad (in other journals of course) to publish pictures showing more detail than the next fellow's whether or not the figures are accompanied by enlightening interpretations of ultrastructure. Consequently, a number of journals have blossomed out with truly exquisite reproductions of photomicrographs showing many previously unappreciated (even unsuspected) ultrastructural features of plant cells and cell products. Many of these which have substantial physiological interest presumably would appear in PLANT PHYSIOLOGY if our halftone methods were up to the task.

Another technique—older than electron microscopy, of uniquely botanical origin, and being elegantly exploited over the last few years—is chromatography. To bring out all the information in a chromatogram is no easy task for the photographer or for the engraver—or for that matter for the pressman. Experts in chromatography usually are biochemists. Most of them are not well versed in the art of photography. They are prone to report their data as r_f values or else to draw facsimiles of their critical chromatograms. (In the latter case the artists' conceptions can hardly be considered original data, and the editor wages a constant battle with some authors who stoutly defend their artistic efforts because they are esthetically superior to photographs of the original chromatograms.) There are indeed chromatographic experts who photograph their spots and who seek good reproduction of most miserable photographs which, unfortunately, are the best quality obtainable. Such authors have no host of special journals to choose from; they have been lulled into accepting halftones which are entirely satisfactory for most purposes but which are only mediocre reproductions of poor contrast photographs of faint spots on chromatograms.

A third area (also recently come into its own) where photographic reproduction may be critical is radioautography. As more sophisticated experiments are designed, important information more and more often is contained in radioautographs which show a great variety of gray shades. Adequate reproduction of such photographs challenges the printer. Here too the scientist may be unhappy when his printed illustrations are less clear than his glossies and thus fail to do justice to his original data.

During the past year or two PLANT PHYSIOLOGY has received an increasing number of manu-

scripts in the areas mentioned; many contain figures which call for better reproduction than we then provided. The editor has learned (both directly & through his spies) of other manuscripts which subjectwise were eminently suitable for PLANT PHYSIOLOGY but which were submitted to a journal whose halftone reproduction was superior to ours. In 1960 PLANT PHYSIOLGY has improved photographic reproduction of selected critical figures. Beginning with the January issue of Vol. 35 we have used finer halftones [(133 mesh) & copper plates] for those photos which the editor designates for special processing. In order to enjoy the full advantage of the finer halftones, beginning with the March issue the Journal has been printed on a better quality (and more expensive) paper. This increases the printing cost slightly more than 5%. As yet we are not charging authors an extra fee for special processing of critical photographs. This temporary policy should not be considered precedent setting.

STATISTICS: One of the more soporiferous tasks of the editorial office is to compile tabular information on each year's pile of new manuscripts. These statistics however provide substantial information on our total operation. The following tabulation includes manuscripts received from 11 August 1959 through 10 August 1960. Apparent discrepancies between certain subtotals are due to some manuscripts appearing in more than one category.

New manuscripts received		185
Accepted for publication		127
With little or no revision	92	
After major revision	35	
Rejected		29
Withdrawn		6
Papers now in process		72
Awaiting assignment to reviewers	13	
In hands of reviewers	20	
Awaiting editor's decision	7	
Awaiting return by author	32	
Papers in press		57
September issue	35	
November issue	22	

TIME STUDY ON MANUSCRIPTS: How long does it take from the time an author submits a manuscript to PLANT PHYSIOLOGY until it appears in print? Normally this has varied between about 7 and 10 months. For the six issues from September 1959 through July 1960 the following averages apply:

From receipt of manuscript to acceptance	6.9 weeks
From acceptance of article to publication	7.5 months
Total time	9.2 months

QUANTITATIVE TRENDS: The Journal is publishing more pages per volume than ever before. Since a two-column format was adopted in 1954 our experience has been as follows:

Vol.	Year	No. articles published	Total pages/vol.
29	1954	113	554
30	1955	104	575
31	1956	101	499
32	1957	127	691
33	1958	92	461
34	1959	124	692
35	1960	153	1,020

During the last Journal year (issues of Sept. 1959 through July 1960) we published 5% more manuscripts and 32% more pages than in the previous six issues. The average manuscript length rose from 5.5 pages to 7.0 pages.

Currently we publish all articles which the editor deems acceptable. The manuscript rejection rate last year was about normal (19%). The length of published articles is held to a minimum partly by editorial encouragement to authors who should condense their manuscripts but also by the brutal device of slapping on a $20.00 per page charge for overrun beyond ten pages. Unless there has been a change in the editor's ability to judge, the longer manuscripts do not represent an increase in flagrant padding but hopefully reflect a trend away from "telegrams" toward more substantial papers. If we do not tighten our editorial tolerances regarding either quality or length of manuscripts perhaps we shall have to accept a somewhat larger journal cost in the future.

Alternatively we could freeze the size of each volume of PLANT PHYSIOLOGY at some arbitrary number of pages. Then the editor would be forced to reject some contributions for lack of Journal space. Presumably the poor papers would be rejected preferentially.

BUDGET: In last year's annual report the editor noted (perhaps smugly) that the actual expenses of his office were within about ½% of his prior estimate. He also felt rather confident that this year's total printer's and editor's expenses would be covered comfortably by the $24,250 which the Executive Committee authorized last August. The following tabulations indicate how reliable was this expectation:

	1959–60		1960–61
	Proposed	Actual	Proposed
Editorial Office			
Editorial Assistant	$ 2,020.	$ 1,874.60	$ 2,150.
Clerk-typist	666.	985.71	1,000.
Editor's stipend	1,000.	1,000.00	1,000.
Postage	300.	503.10	500.
Office supplies	75.	104.91	100.
Telephone & telegraph	50.	47.05	50.
	$ 4,111.	$ 4,515.37	$ 4,800.
Printing and mailing	19,000.	21,930.26.	19,500.
	$23,111.	$26,445.63	$24,300.

We ran a deficit of approximately $2,200. Our operating costs ran 10 % above estimate for the editorial office and 15 % above for printing and mailing. A part of this deficit is accounted for by the fact that the printing and mailing cost represents seven Journal issues—a price we must pay as we begin publishing on schedule. The cost of better quality paper also was a factor. Also important was the increase in size of our Journal. From September 1958 through July 1959 we published 650 pages in six issues. In the corresponding issues for 1959–60 we published 860 pages. The increase was 32 %.

In order to make up a budget for the coming Journal year the editor assumes that we shall publish about 800 pages in our next six issues. This estimate probably is conservative. The editorial office costs are projected on the basis of past experience. The proposed budget for 1960–61 is shown in the far right column of the above table.

Dr. John G. Torrey was appointed by the committee to serve on the Editorial Board.

The secretary, C. O. Miller, reported the nomination and election of new officers. The counting of ballots was carried out by the secretary, Eugene Fox, and David Blaydes. The following were elected:

President—Aubrey W. Naylor
Vice-President—Harry Beevers
Member of Executive Committee—P. J. Kramer
Members of Editorial Board—Harry A. Borthwick
Martin Gibbs

He also reported that abstracts of the Oklahoma State University meetings were printed as a supplement of PLANT PHYSIOLOGY.

Dr. B. S. Meyer presented a report on the activities of the AIBS Governing Board. In accordance with his request, the committee recommended that the Society meet with the AIBS the next two years.

Reports from R. W. Kraus for the National Research Council and M. A. Sprague for the Joint Committee on Grassland Farming were presented.

After several other items were discussed without any new actions resulting, the committee adjourned at a little after midnight.—C. O. MILLER, *Secretary.*

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Business meeting, Oklahoma State University, Stillwater, Oklahoma, 29 August 1960:* President R. H. Burris called the meeting to order at 4:15 pm. There were approximately 60 members present.

The minutes of the previous annual meeting were approved. The minutes of the Executive Committee meeting were summarized and accepted.

The president announced the appointments of W. H. Klein as the new executive secretary-treasurer and J. G. Torrey as a new member of the Editorial Board. Dr. Burris also indicated that a new trustee was being selected.

The executive secretary-treasurer gave a brief report. This was Dr. Noggle's last year in this office and Dr. Burris expressed the thanks of the Society for the fine job which Dr. Noggle had done.

The editor of Plant Physiology, Dr. A. H. Brown, discussed some matters which are detailed in the accompanying minutes of the Executive Committee Meeting. The editorial board has recommended that abstracts continue to be required for short papers to be presented at the annual meetings, and that reading of papers by title only not be permitted.

Dr. Burris discussed the current studies being carried on by a group from the Botanical Society. This study is to explore possibilities of greater integration of botanical groups within the AIBS.

Dr. Martin Gibbs moved that the group strongly recommend that each year the Society sponsor at the annual meeting a symposium consisting of talks on several currently "hot" research topics. The motion carried.

Vice-president W. D. Bonner expressed thanks and appreciation to Dr. Charles Leinweber and Dr. G. W. Todd for making such fine arrangements for the meetings and to Dr. Leinweber, Dr. C. O. Miller, and Dr. Lawrence Rohrbaugh for their efforts as the program committee.

President Burris adjourned the meeting at about 5:15 pm—C. O. MILLER, *Secretary.*

JOHN WESLEY SHIVE*
1877–1960

Our late friend and associate in science, Dr. John Wesley Shive, was born in Halifax, Pa., on Feb. 13, 1877. Preparatory training for college was acquired at a state normal school. He received his baccalaureate in philosophy from Dickinson College at Carlisle, Pa., in 1906, followed by the Master of Arts in 1907 at the same college, specializing in biology, chemistry, and physics with botany as his major subject. From 1906 through 1911 he was an instructor in science at Perkiomen Boys School, at one time also teaching Latin. From 1913 to 1915 he was a research fellow at Johns Hopkins University where he received the Ph.D. degree in Plant Physiology under the direction of Burton E. Livingston. Minor academic subjects included botany, and organic and physiological chemistry. During graduate residence at Johns Hopkins he served as a summer assistant in reasearch at the Desert Laboratory of the Carnegie Institution of Washington at Tucson, Ariz.

In 1915, Dr. Shive was appointed as plant physiologist in the New Jersey Agricultural Experiment Station and lecturer in plant physiology in the Department of Botany at Rutgers. Following the death of Dr. Byron D. Halsted in 1918, a department of plant physiology was formed in the College of Agriculture and Dr. Shive was appointed as its chairman. He concurrently served as chief of the Division of Botany for the men's colleges at Rutgers. He held these administrative offices till the time of his retirement in 1946.

John Shive is probably best known for his intensive pioneering research in the field of mineral nutrition of plants. This development was an outgrowth of his graduate research. Alone, and with associates, he developed techniques for studying the growth of plants in both solution and gravel cultures. Although four or more salts of the major essential elements were used and studied, Shive is noted for the proposed application of a three salt combination, viz., calcium nitrate, potassium dihydrogen phosphate, and magnesium sulfate. By triangular design, each salt added could be varied in proportion to the others such that innumerable variations could be compared. Extensive investigation was made toward seeking a so-called best balance of supply of ions essential to plant development. It was established that fair latitude exists in the balance of ionic supply yielding optimal growth. Several of the combinations of salt supply, which from his study proved favorable, are used extensively by investigators. He devised various techniques for studying plant nutrition from solution and sand cultures, including means of aeration and maintenance of relatively constant composition of the media. Water relations of plants also came under his observation. He was one to consider thoroughly the availability of water to and the wilting phenomenon of plants. He early conceived that the rate of transpiration in some measure determines the observed wilting percentage of the root medium. He recognized the applicability of sand and solution culture methods for developing plants for research as well as for conditions where economics and/or special needs could determine the modus operandi, e.g., "soilless" culture on Ascension Island. At the same time, he was realistic in the latter regard in discerning that such methods could and would merely augment, but not supplant, usual agricultural practice.

Dr. Shive was a deeply penetrating scholar, scorning shallowness; analytical by nature, skillful in developing a line of inquiry and in pursuing productive research to a logical and sound conclusion. He was a grammatical purist, meticulous in the choice of words to most appropriately express his intended meaning. He was an inspiring and conscientious teacher.

John Shive contributed greatly to the training of his graduate students in plant physiology over a quarter of a century; he enhanced their progress through beneficent encouragement. A large proportion of these students continued in research and teaching in various fields both at Rutgers and elsewhere. In cooperative endeavor with students and colleagues, both within his field of study and between disciplines, he recognized their contribution of ideas and effort, sharing modestly joint authorship in publication.

Dr. Shive rose to great heights academically and professionally. Numerous honors were bestowed upon him. He served his campus community as president of the local chapters of Phi Beta Kappa and Sigma Xi and was elected to honorary membership in each. In 1946 he was honored with a Doctor of Science degree from Rutgers University and was recognized in the American Men of Science as an outstanding botanist in 1939 and again in 1944. He was distinguished internationally in 1939 with honorary membership in the Swedish Royal Academy of Agriculture. A man of service, he helped his profession as associate editor of both Soil Science and the journal of the American Society of Plant Physiologists, and officiated as President of this society during the year 1939 to 1940. "In recognition of his outstanding record as an investigator and many years

* The writer is indebted to colleagues of Dr. Shive and to Mrs. Shive for informational materials.

of service in the realm of plant physiology", he received Charles Reid Barnes Honorary Life Membership in the American Society of Plant Physiologists in 1950. The greatest esteem by his colleagues was made manifest with his being awarded the Stephen Hales Prize. The accompanying citation of the plant physiology society expressed succinctly their regard of this professional man: "For nearly 25 years associated with the investigation of the mineral requirements of plants; originator of new and of improved methods for growth of plants in culture under controlled conditions; one of the earliest to appreciate the fundamental relationship of hydrogen-ion activity of the culture solution to the absorption of the inorganic ions by the plant and to develop practical methods of plant culture based upon these relationships; student of the chemical composition of plant tissues as affected by the nature of the culture solution; inspiring teacher and leader of a devoted corps of younger investigators; a man whom this society is proud to have among its membership and a scientist whose singlemindedness of purpose and definiteness of aim have led him to achievement of highest caliber."

Dr. Shive was a true gentleman. He had a delightful combination of seriousness about his work and at the same time an appreciation of the interesting and humorous. He listened well, thought much, and talked sparingly. He was warm-hearted and considerate of everyone.

Physically, he was blessed with good health and a strong constitution. He was fond of outdoor life, enjoying swimming, hiking, and camping with his sons (John and Scott), and later with his grandchildren. For many years his principal recreation during the summer season was a daily game of tennis. For some years before his retirement, his vacation comprised a camping trip to one of the western national parks. On these trips, he took series of extraordinarily beautiful and interesting color slides of plants, animals, and scenic views. These he generously shared with many audiences. During retirement years, he developed a new hobby, that of deep-sea fishing. This so enthralled him that he and his wife moved to Barnegat on the New Jersey shore where he could operate his boat in the bay, and from where he could launch out further on charter boats. Death came to him through a heart attack, on June 22, 1960, at the age of 83 years, while setting out with his boat for a fishing trip in Barnegat Bay.

Dr. John Wesley Shive was an enthusiastic and outstanding contributor to the developing field of plant physiology during a large part of the first half of this century, a great credit to the profession. He exemplified a life of serious endeavor, enquiry, and giving for others, balanced by a happy home life, with geniality, relaxation, and communion with nature.—T. C. Broyer.

Walter Ferdinand Loehwing
1896–1960

Dean Walter F. Loehwing, Professor of Botany and Dean of the Graduate College of the University of Iowa, died at home on August 1, 1960. His unexpected death was attributed to heart failure.

Walter Loehwing was born Nov. 25, 1896, in Chicago, Ill., a son of Charles Henry and Emilie Hildebrandt Loehwing. While an undergraduate at the University of Chicago, he enlisted in the citizens' army of World War I and served with artillery in France. He was exposed to gas warfare and bore the scars and damage of this experience the rest of his life. After the war he attended the Pasteur Institute in Paris and received a certificat superieur, alliance Francaise. Returning to the University of Chicago he received the B.S. degree in 1920, the M.S. in 1921, and the Ph.D. magna cum laude in 1925.

He was among the first group of graduate students to work with Prof. Charles A. Shull who describes him at that time as "a vigorous worker with ability to plan and execute his research". His investigations required many Kjeldahl determinations and his sensitivity to acid fumes emphasized the inadequacy of the old botanical laboratory for such work and ultimately led to the inheritance of the Koch laboratory by Prof. Shull and his students, which was used until the Barnes Laboratory of Plant Physiology was built. During his recuperation he held his first teaching position at the Oklahoma State School of Mines from 1921 to 1923 where he was professor of chemistry and head of the department.

With the Ph.D degree in botany he came to the attention of the late Prof. R. B. Wylie, also a Chicago doctorate in botany, and was given the assignment of developing plant physiology at the University of Iowa in 1925. He rapidly established his reputation as an effective teacher of general botany, genetics,

and physiology. A syllabus for his course was a masterpiece of detailed organization and dynamic presentation. He was also very active in research, and published papers on mineral nutrition, root and soil interactions, and plant tissue fluids at this time. He became Professor of Botany in 1930 and his expanding teaching and research programs and those of the students working with him soon required additional laboratory and greenhouse facilities. By considerable ingenuity and perseverance he was able to arrange for the construction of a new laboratory and greenhouse with half the funds provided by the federal WPA and half by the state. This building was designed generally for teaching and research in experimental botany and when constructed in 1936 it ranked among the finest such laboratories in existence. Mineral nutrition continued to be the principal research interest of Loehwing and the graduate students working with him, but investigations of the physiology of sex, photoperiodic response, and developmental physiology were also published in the period of 1937 to 1942. His address as vice president and chairman of the Section of Botanical Sciences of the A.A.A.S., "Developmental Physiology of Seed Plants," in 1947 dealt with these studies and their implications and was his final scientific contribution. The laboratory for plant physiology was destroyed in 1948 to clear space for a Veterans Administration Hospital.

In addition to the talents of Dean Loehwing in teaching and research he was also a very able administrator. He was made head of the botany department in 1940 and continued in the position until 1953. In 1950 he was chosen by the faculty to be Dean of the Graduate College to which office he brought a broad understanding of scholarship and a considerable sympathy for the problems of the academic researcher. Through the years of World War II and afterward he served on many special committees concerned with changes in college curricula in biology and the training of teachers and he published a number of articles on these subjects. Recently he had completed the planning of a special master's degree program supported by the Ford Foundation to strengthen the preparation of students for college teaching.

Dean Loehwing's death has been described as coming at the height of his power in academic administration. He had served as president of the Association of Graduate Schools in the American Association of Universities from 1958 to 1959 and was serving as acting secretary of that organization. He was a member of the advisory committee of the National Science Foundation and the advisory committee of the U.S. Office of Education on Title IV of the National Defense Education Act. From 1956 to 1957 he was chairman of the Midwest Conference on Graduate Study and Research.

He was a member of numerous organizations including 12 scientific societies in many of which he had held office. He was a lieutenant governor of Kiwanis for the Iowa-Nebraska district in 1943. He was president of the Iowa Academy of Science from 1956 to 1957 after serving earlier as treasurer and vice president and was vice president of the Botanical Society of America from 1946 to 1947.

Walter Loehwing's contributions and efforts in behalf of the American Society of Plant Physiologists are widely known and appreciated. Prof. Shull, the first president of the Society, writes, "He was a friend of the Society from the very beginning and was one of the young men who gave me the courage to accept the presidency of ASPP. He helped me to see what a strong society would mean to the welfare of Plant Physiology, the science". Loehwing was secretary-treasurer of ASPP from 1935 to 1937, vice president from 1937 to 1938, and president from 1938 to 1939. He became an editor of PLANT PHYSIOLOGY in 1939 and succeeded Prof. Shull as editor-in-chief in 1945. Like his predecessor he gave freely of his time and finances to promote the success of the Journal until 1953. He received the Charles Reid Barnes Life Membership Award of the Society in 1955.

On February 26, 1936, he was married to the former Helen Cromer, who survives him.

Dean Loehwing was kindly, generous, and considerate toward all. In the tributes of his associates his loyalty to ideals, his university, his profession, colleagues, and students is given signal mention.—ROBERT M. MUIR.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

OFFICERS FOR 1961

President: Dr. Aubrey W. Naylor
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Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Correspondence concerning membership and its privileges, dues, change-of-address, etc., should be addressed to the Executive Secretary-Treasurer, Dr. W. H. Klein, Div. of Radiation and Organisms, Smithsonian Institution, Washington 25, D. C. Business correspondence regarding subscriptions, back numbers, change-of-address of subscribers, etc., may be addressed to the Executive Secretary-Treasurer at the address above. All matters pertaining to nominations, elections, programs, and meetings of the society should be addressed to the Secretary, Dr. C. O. Miller, University of Indiana, Bloomington, Indiana. Correspondence with reference to editorial matters should be addressed to the Editor, Dr. Allan H. Brown, Department of Botany, University of Minnesota, Minneapolis 14, Minnesota.

INSTRUCTIONS FOR CONTRIBUTORS TO PLANT PHYSIOLOGY

I. WRITING MANUSCRIPT.

A. STYLE. In general, the style of an article will reflect the author's preference and habits of writing. The Editor may suggest improvements, but will not insist on rigid conformity to conventions at the expense of an author's individuality. Scientific writing, however, should meet accepted standards of grammar, syntax, and diction.

Strive for specific, concise expression. Use active verbs. Avoid complicated sentence structure. Write in a straightforward manner. Prepare the original manuscript carefully to obviate subsequent delays in publication because of the need for manuscript revision.

B. LENGTH. Beyond ten pages (including tables, figures, & literature citations) authors will be billed $20 per page for overrun. Approximately four double spaced typewritten pages (pica type) equal one page of printed type.

C. FOOTNOTE REFERENCES. These should be avoided in the body of the text, but if a footnote must be used, it should be numbered with an Arabic numeral and typed at the bottom of the page on which reference to the footnote is made.

D. ACKNOWLEDGMENTS. Do not include acknowledgments in a footnote; they belong under their own heading following the SUMMARY and preceding LITERATURE CITED.

E. PUNCTUATION.

1. Periods and Commas. These are the most often used and misused punctuation marks; the period is not used enough, the comma too often. Avoid complicated sentences which require many commas, colons, or semicolons. Break such long sentences into brief, concise sentences which will be more easily understood.

2. Quotation Marks. Avoid the urge to use excessive quotation marks. Often a word is used in a sense slightly different from its ordinary meaning. This does not necessarily warrant the use of quotation marks. In case a term is used in an unconventional way and the ambiguity might lead to misunderstanding, use quotation marks only when the unfamiliar meaning is first explained.

3. Brackets. Material is customarily enclosed in single brackets: ( ). Where double brackets are necessary use the following: [( )]. Abbreviations are the rule: &, fig, hr, min, sec.

4. Hyphens. Use sparingly.

F. SUBTOPICS. Use this order and style to number subtopics:

I. I)
A. A)
1. 1)
a. a)

G. SCIENTIFIC NAMES. The complete botanical name (genus, species, & authority for the binomial) of all experimental plants should be included, under MATERIALS AND METHODS. Refer to the most recent International Code of Botanical Nomenclature for rules governing the use of scientific names.

In general PLANT PHYSIOLOGY follows the style recommenations of the Conference of Biological Editors—American Institute of Biological Sciences, Committee on Form and Style. 1960. Style manual for biological journals. Am. Inst. Biol. Sci., Washington.

H. ABBREVIATIONS.

In place of certain unwieldy chemical names abbreviations may be used as a convenience. The writing, however, must remain readily comprehensible and unambigious. For the most part only widely used abbreviations should be employed (e.g.: ATP, RNA,). Standard chemical symbols may be used without definition: Ca, NaOH. All others should be defined when they first appear. Example: The reaction was thought to involve flavin adenine dinucleotide (FAD). If your article uses several abbreviated forms it is permissible to define them all in a single footnote where the first abbreviation is introduced.

In no case should the number of nonstandard abbreviations be so large that the reader is forced to learn a special vocabulary in order to read your article easily. Abbreviations should not frustrate the reader; they should be used for his convenience, not the author's convenience.

In title and summary avoid abbreviations which are not generally accepted. Because titles and summaries frequently are translated into foreign languages, undefined abbreviations may be confusing.

I. CHEMICAL FORMULAE. It is expensive (sometimes impossible) to set formulae in type. To avoid errors, submit structural formulas or involved reaction sequences as clearly executed India ink drawings which can be photographed. Indicate where these are to be inserted into the

text. Alternatively a complex reaction diagram or flow sheet may be treated as a regular figure, given a number, and supplied with a legend.

II. PREPARING TYPESCRIPT.

Use bond paper for the first copy in preference to onion skin. Consult recent editions of PLANT PHYSIOLOGY for proper placement of main headings, paragraph headings, and subheadings.

A. SPACING. Double space EVERYTHING, including tables, legends, literature cited, and footnotes. It is essential that all copy be double spaced to allow room for the extensive proofmarking which must be done (even to a perfect typescript) in the editorial office so the printer will know precisely how to set your copy in type.

Begin typing 1/3 of the way down the first page; leave liberal side margins.

Double space each table, each set of figures, each set of legends, and each set of literature citations on a separate page; these items are given by the printer to different people for setting in type sizes different from that in the body of the manuscript.

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C. PAGING. Number all pages consecutively (including tables & legends) in upper right hand corner. Never use a letter such as 2a, 5D, for paging: such pages are easily lost during processing.

D. PAGE ONE. Do not use a title page.

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2. *Footnotes.* One or more footnotes are included at the bottom of the first page. The first footnote (indicated by superscript 1 after the title) gives the date the manuscript was received. The second footnote credits the source of financial support (if any). Additional footnotes give the present address of the author, if different from the address in the heading, and indicate the experiment station or institution paper number. Normally these are the only footnotes used.

E. NUMBERS.

1. Write out all numbers (cardinal & ordinal) from one through ten. Use figures for all numbers larger than ten. Exceptions:

a. When using precise measurement such as 5 per cent, 6 mm, 8-hour cycle, figure 1. In nonprecise usages spell numbers out: about two weeks, six or eight degrees.

b. In a series of related numerical expressions treat all alike even though violating the general rule: 2 leaves, 8 leaves, and 12 leaves.

c. When a single expression has consecutive numbers, use both figures and spelled out numerals: two 6-minute exposures.

d. Spell out ALL numbers or fractions which begin a sentence. If this is awkward, rephrase the sentence to avoid beginning with a numeral.

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F. FRACTIONS. Write out and hyphenate nonprecise simple fractions: two-thirds, *but* half strength. Exceptions: tables, figures, graphs, legends, fractions in parentheses. Often it is desirable to use decimals instead of fractions. For values less than one, insert a zero before the decimal point: 0.7 g *not* .7 g.

G. LITERATURE CITED. This section of a manuscript requires extensive proofmarking even though copy is correct in every detail. Typing errors are easily made, but difficult to detect, so exercise special care in preparing and checking the bibliography. Since they are set in a typeform different from that used for the body of the manuscript, literature citations should be *double spaced on a separate piece of paper.* Consult the most recent issue of PLANT PHYSIOLOGY for Journal style. Note the limited use of capitals.

Check all citations (such as title, volume number, pages, & date) for completeness. Check foreign titles for diaresis and other markings. Make certain that all citations are referred to in the text.

List citations in alphabetical order (not in order of reference), then number them and indicate citations in the body of the text by Arabic numerals enclosed in parenthesis.

Unpublished data, personal communications, and articles in preparation are not acceptable as literature citations, but should be referred to parenthetically in the text. Articles that are in press may be so designated in LITERATURE CITED. An article is not properly referred to as "in press" unless it has been *accepted* for publication. The journal in which an in press article will appear should be included in the literature citation.

1. Authorship. List family name of first or sole author before listing initials or given name. Thereafter write names and initials (or given name) of all coauthors in their natural order. Use initials for a man's given name; write out a woman's given name: Reeves, A. C., R. D. Jones, and Helen Smith.

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On the average, about 52 typewriter spaces (excluding the new executive typewriter) fill one 3-inch line of type of the size normally used in tables: ten double spaced typewritten lines require 1 inch of the available vertical printed space.

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C. FOOTNOTE DESIGNATIONS. Refer to footnotes by the asterisk, double asterisk, triple asterisk, dagger, double dagger, and section, in this sequence as needed: *, **, ***, †, ‡, §. *Place all explanatory material in footnotes* rather than in the heading or columnar headings, both of which should be as *brief as possible* without sacrificing clarity.

D. HEADINGS & FOOTNOTES. Wherever possible, omit unnecessary articles such as "the", "a", in headings and notes. Also avoid bulky prepositional phrases.

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Guided lettering is preferable to hand lettering or typing.

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Limit the area of each photograph to that which is necessary to show pertinent details. Trim off irrelevant portions and minimize blank space.

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Send the original copy of the manuscript, the first carbon, and two copies of all illustrative material to the Editor, Dr. Allan H. Brown; Department of Botany; University of Minnesota; Minneapolis 14, Minn. Retain a carbon copy of your manuscript and original photographs or drawings to insure against loss.

PLANT PHYSIOLOGY does not accept a manuscript which has not been submitted directly by an author. If your manuscript is submitted by someone other than one of the authors, send a letter to this office stating that you wish to publish that particular manuscript in PLANT PHYSIOLOGY. Your manuscript cannot be processed until this letter is received. This precaution protects you as well as the Journal.

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A. REVIEW. The manuscript usually will be sent to two reviewers familiar with that field of research covered by the paper. Each reviewer evaluates the manuscript, suggests improvements, and recommends accepting or rejecting the paper. Usually two months suffice for review and decision. When there is marked disagreement between reviewers, the Editor may appoint a referee (often a member of the Editorial Board) to evaluate the manuscript in light of the reviews; the referee recommends either for or against acceptance.

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Retype each page on which there are extensive changes.

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a. Proofread Manuscript. Check ALL references carefully BEFORE submitting manuscript to Editor. Corrections in typing are easy to make, whereas corrections in galley or page proof are expensive and sometimes difficult to make.

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